



Selenium metabolism

Intestinal and hepatic metabolism of selected selenium compounds

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Preface

The present thesis is submitted to meet the requirements for attaining the Ph. D. degree at The Faculty of Pharmaceutical Sciences, University of Copenhagen, Denmark.

The experimental work was performed at the Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen with Associate Professor, PhD Bente Gammelgaard, Primary ADME Scientist, PhD Lars Bendahl and Professor, PhD Bent Halling-Sørensen as supervisors.

The main part of the experimental work of the PhD project has been published or submitted for publication in relevant scientific journals and copies of the papers are included in Appendices I-III. Furthermore, involvement in a manuscript on speciation of selenium compounds related to human selenium metabolism has led to co-author status on a review which is included in Appendix IV. This thesis gives an introduction, discussion and conclusion of the results obtained. Published results will be referred to as *papers I-IV*. Whenever relevant to the discussion, produced results that are not presented in the publications will be included. For details of the published studies the reader is referred to the appendices.

- I. Gabel-Jensen C, Gammelgaard B, Bendahl L, Stürup S and Jøns O. Separation and identification of selenotrisulfides in epithelial cell homogenates by LC-ICP-MS and LC-ESI-MS after incubation with selenite. *Anal. Bioanal. Chem.* 2006; 384: 697-702
- II. Gabel-Jensen C, Lunøe K, Madsen KG, Bendix J, Cornett C, Stürup S, Hansen HR and Gammelgaard B. Separation and identification of the selenium-sulfur amino acid S-(methylseleno)cysteine in intestinal epithelial cell homogenates by LC-ICP-MS and LC-ESI-MS after incubation with methylseleninic acid. *J. Anal. At. Spectrom.* 2008; 23: 727-732
- III. Gabel-Jensen C, Odgaard J, Skonberg C, Badolo L and Gammelgaard B. LC-ICP-MS and LC-ESI-(MS)ⁿ identification of Se-methylselenocysteine and selenomethionine as metabolites of methylseleninic acid in rat hepatocytes. (*Manuscript submitted for publication*)
- IV. Gammelgaard B, Gabel-Jensen C, Stürup S, Hansen HR. Complementary use of molecular and element-specific mass spectrometry for identification of selenium compounds related to human selenium metabolism. *Anal. Bioanal. Chem.* 2008; 390: 1691-1706

During my enrolment as a PhD student I spent three month in the laboratory of Assistant Professor, PhD Angeline S. Andrew at Dartmouth Medical School, Section of Biostatistics and Epidemiology, Hanover, New Hampshire, USA. Alteration of genetic pathways in a human bladder cancer cell line treated with methylseleninic acid was investigated by use of cDNA microarray and RT-PCR technologies. The work was outside the specific scope of this thesis and it is not further presented.

Acknowledgements

First of all I would like to thank my main supervisor Associate Professor Bente Gammelgaard inspiring and valuable guidance throughout the project. Thank you for introducing me to the exiting world of biological selenium speciation.

To the *Analytical Chemistry - Bioinorganic Chemistry Research Group* that just keeps expanding; thank you for widespread (scientific) conversation and for taking an interest in my project. Thanks to the master students that participated in the project during the years.

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List of Abbreviations

Cys	Cysteine
EI	Electron Impact
ESI	Electrospray Ionization
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione, reduced form
GS-Se-SG	Selenodiglutathione
GS-Se-Cys	Mixed selenotrisulfide of glutathione and cysteine
GS-SG	Glutathione, oxidized form
HCys	Homocysteine
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
<i>In situ</i>	At the spot
<i>In vitro</i>	Outside the living organism (in test tubes)
<i>In vivo</i>	In the living organism
<i>iv</i>	intravenously
IVD	<i>In vitro</i> digestion
LC	Liquid Chromatography (short for HPLC)
MeSeA	Methylseleninic Acid
MIMS	Membrane Inlet Mass Spectrometry
MS	Mass Spectrometry
<i>m/z</i>	Mass-to-charge ratio
ROS	Reactive Oxygen Species
SeMet	Selenomethionine
Se-MeSeCys	Se-methylselenocysteine
S-(MeSe)Cys	S-(methylseleno)cysteine
S-(MeSe)SG	S-(methylseleno)glutathione
SPS	Selenophosphate Synthase
SRM	Selected Reaction Monitoring
TIC	Total Ion Current

Summary

Selenium is an essential trace element that exerts its effect via a number of selenoproteins. Selenoproteins are mainly involved in redox processes. Furthermore, several studies on cell lines, animal models and human intervention trials have shown cancer protective effects of selenium.

Selenium metabolism is not fully elucidated and there is need for further research in this field as understanding of selenium metabolism may lead to better understanding of the cancer protective mechanism of selenium. So far, selenium excretion in human urine has been extensively investigated and the most important metabolites have been identified as selenosugars. Other end products of selenium metabolism are volatile species excreted via the breath. The aim of this PhD project was to investigate earlier stages in the metabolic pathways. *In vitro* experiments with an intestinal metabolism model were performed in order to investigate if selenium compounds are metabolized intestinally before being available to hepatic metabolism and here after to the whole organism.

The metabolism of selenite and methylseleninic acid was studied in homogenized intestinal epithelial cell from pigs. The major selenium-containing metabolites in the supernatant of the incubated cells were detected by LC-ICP-MS and identified by LC-ESI-MS, either directly in the supernatant or after purification by preparative chromatography. Both species were reduced by glutathione and cysteine, the major thiol compounds present in gastric and intestinal lumen. The reduction of selenite and methylseleninic acid was spontaneous and did not require enzymatic activity. Neither of the reduction products identified have been identified in mammalian intestinal models before and the findings emphasize that selenite and methylseleninic acid are not bioavailable in their intact forms.

Methylseleninic acid, which is often used as a model compound for methylated selenium amino acids, was also incubated in isolated rat hepatocytes to investigate hepatic metabolism of this species. Parallel to the intestinal model studies, metabolites excreted from the hepatocytes were detected by LC-ICP-MS and identified by LC-ESI-MS after purification by preparative chromatography and pre-concentration by lyophilisation. One major metabolite of methylseleninic acid was Se-methylselenocysteine, the same methylated selenium amino acid to which methylseleninic acid is considered a model compound. The metabolic findings in the intestinal and hepatic models combined indicate that methylseleninic acid may not be a relevant model compound for methylated selenium amino acid. Another metabolite was selenomethionine which is widely used in selenium intervention trials.

Dansk Resumé

Selen er et essentielt grundstof, som udøver sin funktion via selenoproteiner, der hovedsageligt indgår i redoxprocesser. Endvidere har flere forsøg i celler, dyrmodeller og humane studier vist at selen har en beskyttende virkning mod kræft.

Selens metabolisme er ikke fuldt verificeret. Opklaring af selens metabolisme er vigtig som led i forståelsen af den kræftbeskyttende virkning. Hidtil har meget forskning været fokuseret på identifikation af slutprodukter i urin, og de vigtigste metabolitter er nu fastslået at være selenosukker. Andre slutprodukter i selenmetabolismen er små flygtige forbindelser, der udskilles via åndedrættet. Formålet med dette Ph.D. projekt var at undersøge tidligere trin i metabolismen. *In vitro* forsøg i en tarmmodel blev udført for at undersøge om relevante selenforbindelser metaboliseres før de optages og føres til leveren, for derefter at blive systemisk tilgængelige.

Selenit og methylseleninsyre metabolisme blev undersøgt i homogeniserede epitelceller fra en svinetarm. Hovedmetabolitterne i supernatanten fra cellehomogenatet blev detekteret med LC-ICP-MS og identificeret med LC-ESI-MS enten direkte i supernatanten eller efter oprensning med præparativ chromatografi. Både selenit og methylseleninsyre blev reduceret af glutathion og cystein som er thiol, der findes i relativ stor mængde i mave- og tarmvæske. Reaktionen forløber spontant og er ikke enzymatisk betinget. Reduktionsprodukterne, der kaldes selenotrisulfider og methylselenylsulfider, er ikke tidligere identificeret med LC-ESI-MS i tarmmodeller og resultatet tydeliggør, at selenit og methylseleninsyre ikke er biotilgængelige i deres native form.

Methylseleninsyre, der ofte anvendes som modelstof for methylerede selenaminyrer, blev yderligere inkuberet med isolerede leverceller fra en rotte. Leverceller anvendes som model for metabolisme i leveren. Ekstracellulære metabolitter blev detekteret med LC-ICP-MS og identificeret med LC-ESI-MS efter oprensning med præparativ chromatografi og opkoncentrering med frysetørring. Methylseleninsyre omdannedes til Se-methylselenocystein, den samme selenaminyr, som det anvendes som modelstof for. Resultatet fra tarmmodellen og levermodellen tilsammen sætter spørgsmålstegn ved om methylseleninsyre er et relevant modelstof for methylerede selenaminyrer. Selenomethionin, der er den forbindelse, der ofte anvendes i interventionsstudier, blev også identificeret som metabolit af methylseleninsyre.

1 Introduction

1.1 Selenium Chemistry

The common oxidation states of selenium are -2, 0, 4 and 6. In biological samples the most common oxidation state of selenium is -2 in forms of the protein-bound selenoamino acids selenocysteine (SeCys) and selenomethionine (SeMet). Biological samples contain other divalent selenium compounds such as methylated selenoamino acids and methylated forms of hydrogen selenide. Inorganic compounds with selenium in high oxidation states such as selenite (+4) and selenate (+6) are rarely found in biological samples. Selenium and sulfur compounds share some structural similarities but differ widely in chemical properties for example selenols are stronger acids than their related thiols and compounds with selenium in the -2 oxidation state are more reducing while compounds with selenium in the +4 and +6 oxidation state are more oxidizing than the related sulfur compound[4]

1.2 Selenium and health

Selenium is an essential trace element in humans and selenium deficiency can have adverse consequences for disease susceptibility and maintenance of optimal health[5]. Selenium functions through diverse physiological pathways via selenoproteins. A number of selenoproteins have been identified, however the function of all of them has not yet been determined[6,7]. The most abundant selenoproteins are involved in general defence against oxidative stress. Selenium has immunostimulant effect and selenium deficiency is linked to occurrence, virulence and disease progression of some viral infections. It is essential for male fertility and thyroid function[5].

Since the publication of the first double-blind, placebo controlled intervention trial in which selenium supplementation caused reduction in overall cancer mortality and reduced the number of incidences of lung, prostate and colon cancer[8], selenium has gained much interest as a cancer preventive agent.

Common sources of selenium are selenomethionine (SeMet) and selenocysteine (SeCys) from vegetable and animal food. Nutritional supplements represent another source of selenium that often contains selenite, selenate, SeMet and selenium enriched yeast. Recommended daily intake is 50-70 µg/day which is considered adequate to prevent deficiency symptoms and keep the selenoproteins functioning[9].

1.2.1 Selenium and cancer

The cancer preventive mechanism of selenium is not understood; however the degree of protection is highly dependent on the chemical form of the ingested selenium, most likely because different

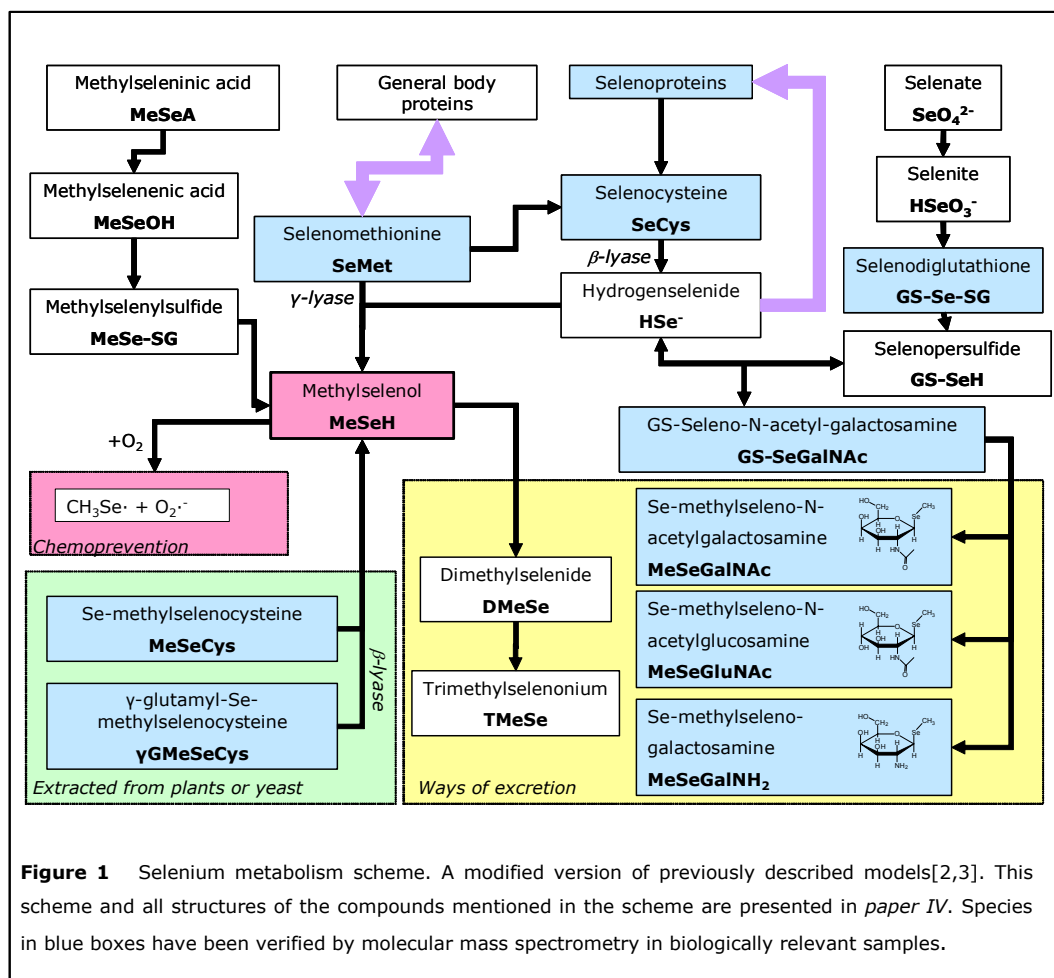
selenium compounds are metabolized in different pathways by the organism[10]. As DNA mutations caused by high cellular concentrations of reactive oxygen species (ROS) may lead to carcinogenesis[11] and antioxidants as general protectors against induced carcinogenesis is currently discussed[12], the antioxidant selenoproteins may play a role in the cancer preventive effect of selenium. However, in cancer intervention trials, doses are 3-4 times higher than the recommended daily intake; hence the cancer preventive effect might not be exerted entirely via the selenoproteins. A theory for the cancer preventive mechanism of selenium in which methylselenol (MeSeH) is a key metabolite has been suggested[13] and selenium compounds that are metabolized into MeSeH are considered to be efficient in cancer prevention. According to this theory Se-methylselenocysteine (Se-MeSeCys) which is believed to produce MeSeH when cleaved by the β -lyase enzymes and methylseleninic acid (MeSeA) which is believed to produce MeSeH by a series of reductions by thiols such as cysteine and glutathione[2] would be efficient cancer protective agents. Another theory of the cancer preventive mechanism of selenium is that selenium compounds in oxidation state +4 are most effective as they can inactivate critical cellular enzymes by oxidizing their sulfhydryl groups and thereby induce apoptosis[14]. According to this theory selenite and MeSeA would be efficient cancer protective agents. Interestingly, the most efficient cancer preventive species according to the proposed theories do not include selenomethionine, the principal species used in intervention trials.

1.3 *Selenium metabolism*

Selenium metabolism is most often described by different variations of a model originally proposed by Ganther[3]. This model is generally accepted although not all steps have been verified. A version of the selenium metabolism model is shown in figure 1.

The overall idea of the metabolism model is that all ingested selenium is metabolised into hydroselenide (HSe^-). HSe^- is then used for further incorporation into selenoproteins or into metabolic end products for excretion[3]. The metabolic steps leading to formation of HSe^- however have not been established and neither HSe^- nor the involved metabolites have been verified.

The degree of bioavailability of selenium is species dependent and dependent of the nutritional source and on the selenium status of the subject[15]. Selenium bioavailability is often referred to as the ability to replete tissue selenium levels or as the activity of the selenoprotein glutathione peroxidase (GPx)[16]. In a review on the bioavailability of selenium from foods, Finley argues that other measures of selenium bioavailability that is not entirely related to selenium tissue levels or GPx activity may be relevant, for example as the ability to reduce cancer[16]. Despite the variability introduced due to the factors mentioned above, an overall bioavailability of all forms of selenium of 70-95 % was reported and selenium from all nutritionally relevant compounds is bioavailable[16].



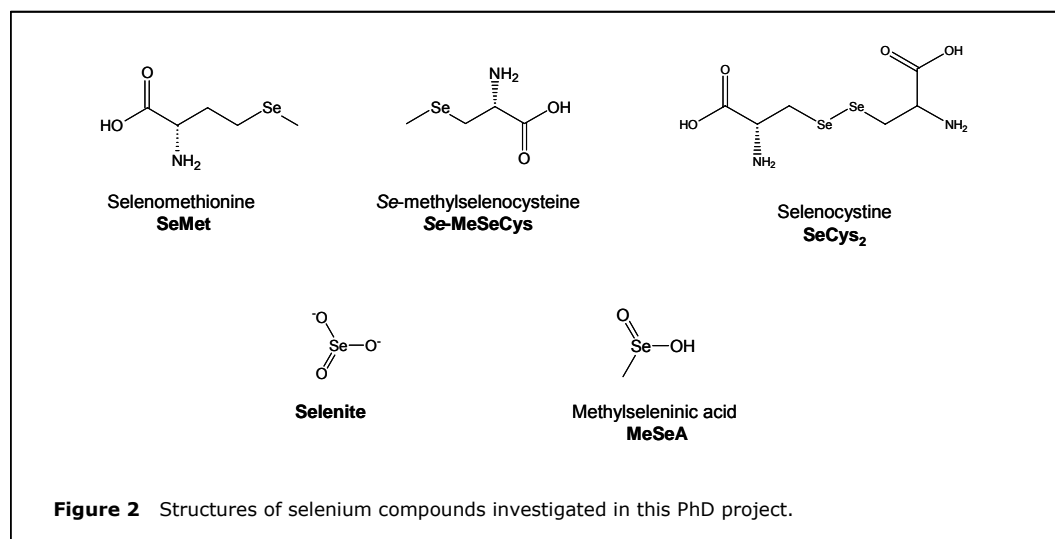
Selenium is specifically incorporated into selenoproteins as SeCys. SeCys residues are often key elements in selenoprotein functional sites. Selenium is also found in all other proteins, where it is unspecifically incorporated as SeMet in competition with the sulphur analogue methionine as the organism is not able to distinguish between these amino acids[17,18]. Opposed to SeCys, SeMet is not crucial for protein activity. As the body is able to use selenium from SeMet to synthesize SeCys for incorporation into selenoproteins, it has been suggested that incorporation of SeMet into the general body proteins serves as a storage function for selenium[19].

Selenium is excreted via urine or breath. The main urinary end product of selenium metabolism is now identified as an selenosugar: Se-methylseleno-N-acetylglactosamine[20,21]. Two other selenosugars have been identified as minor urinary metabolites[22]. The trimethylselenonium ion

(TMeSe) was formerly regarded as the main excretory species of excess selenium; however it has been shown that it is only a minor constituent in urine even after ingestion of large amounts of selenium. The controversial presence of TMeSe in urine has been thoroughly reviewed by Francesconi and Pannier[23]. Another end product of selenium metabolism is the volatile species dimethylselenide (DMeSe) which is excreted via the breath[24,25]. The end products seem to be common end products of selenium metabolism independent of ingested species.

1.4 Aim and rationale of study

Based on the above described selenium metabolism and cancer protection models it is evident that establishment of every step in selenium metabolism of nutritionally relevant selenium compounds is important as it may lead to a better understanding of the cancer protective effect. Metabolism of SeMet, Se-MeSeCys, selenocystine (SeCys₂), selenite and MeSeA was investigated. Their structures are shown in figure 2.



The aim of this PhD project was to identify missing steps of selenium metabolism in order to assess the selenium species available for absorption. *In vitro* models of animal origin are easy and ethical to use and by choosing the right model metabolic findings are likely to be similar to *in vivo* human metabolic findings. Gastro-intestinal and hepatic models were chosen to elucidate initial steps in selenium metabolism as selenium is administered orally.

An overview of the *in vitro* models and the investigated selenium compounds is given in table 1.

Table 1 *in vitro* models and the investigated selenium compounds

<i>In vitro</i> model		Selenium compound investigated	
Model for gastro-intestinal digestion	Simulated gastric and intestinal juices	Selenite, SeMet, SeCys2, Se-MeSeCys	Unpublished
Model for intestinal metabolism	Intestinal epithelial cell homogenates (Pig)	Selenite, MeSeA, (SeMet, Se-MeSeCys)	Paper I and II
Model for hepatic metabolism	Isolated hepatocytes (rat)	MeSeA	Paper III

2 Analytical methods

The analytical challenge in metabolism studies is related to the often very complex matrices of the samples and the low concentration of the metabolites of interest. The procedure of choice in bioinorganic speciation studies is a combination of hyphenated techniques such as liquid chromatography (LC) coupled with inductively coupled plasma (ICP) mass spectrometry (MS) and LC coupled with molecular MS[26].

Although the coupling of LC with ICP-MS is not entirely straightforward[27], the combination of efficient separation of sample components and the good sensitivity offered by the element specific ICP-MS detector provides an excellent tool for detection of selenium containing compounds and LC-ICP-MS is used through out this PhD project to screen samples for selenium metabolites.

Molecular MS provides the structural information that is inherently lost upon LC-ICP-MS analysis. And from the beginning of this millennium it became the analytical approach of choice for elemental speciation to detect species by elemental analysis followed by structural identification of the detected species by molecular MS[28]. A thorough review of the use of molecular MS in speciation analysis was given by Rosenberg[29].

The scope of *paper IV* was to give an overview of the selenium compounds related to selenium metabolism that was identified by the complementary use of element- and molecular specific mass spectrometry analysis.

2.1.1 ICP-MS

ICP-MS is often used as an element specific detector in selenium speciation studies. The use of ICP-MS for selenium detection was thoroughly reviewed by B'Hymer and Caruso[30].

In ICP-MS the liquid sample is introduced into the instrument via a nebuliser and a spray chamber. The nebuliser provides an aerosol and the spray chamber ensures that only the smallest droplets reach the argon plasma. In the plasma, the droplets are desolvated, molecules are decomposed into atoms and finally the atoms are excited and ionized (figure 3). The ions are passed through the mass analyzer and detected based on their mass to charge ratio (m/z)[31]. As all molecules are decomposed to their atomic components no molecular structural data are obtained by ICP-MS.

ICP-MS is considered a rather selective detector; however occurrence of spectral interferences compromises the selectivity. Spectral interferences are caused by atomic and polyatomic ions, formed in the plasma, with the same m/z as the ion of interest. Although selenium has six isotopes, all of them are subjected to interferences. The best known interferences on the selenium

isotopes are shown in table 2. The polyatomic interferences are caused by the plasma gas (argon) or atmospheric gases, the solvent or the matrix of the sample. The most abundant selenium isotope ^{80}Se is severely interfered by the $^{40}\text{Ar}^{40}\text{Ar}^+$ dimer making this isotope practically useless to ordinary ICP-MS. In all ICP-MS analyses performed during this PhD project, the isotopes ^{77}Se , ^{78}Se and ^{82}Se were measured simultaneously. Only the ^{82}Se isotope is presented in the figures because this isotope resulted in the best signal-to-noise ratio. All three selenium isotopes were measured however in order to conclude, based on the relative isotope ratios versus the theoretical ones, whether a signal is specific to a selenium compound or due to an interference.

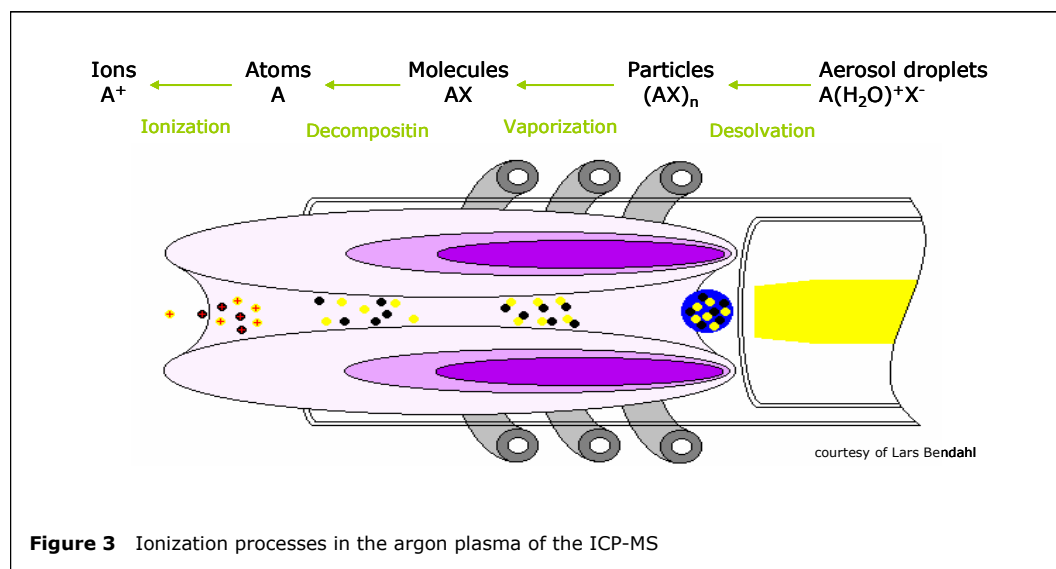


Table 2 Relative abundances and some interferences on selenium isotopes

Isotope	Abundance (%)	Interferences
^{74}Se	0.89	$^{37}\text{Cl}_2^+$, $^{40}\text{Ar}^{34}\text{S}^+$
^{76}Se	9.36	$^{36}\text{Ar}^{40}\text{Ar}^+$, $^{38}\text{Ar}^{38}\text{Ar}^+$, $^{40}\text{Ar}^{36}\text{S}^+$, $^{31}\text{P}_2^{14}\text{N}^+$
^{77}Se	7.63	$^{40}\text{Ar}^{37}\text{Cl}^+$, $^{40}\text{Ar}^{36}\text{Ar}^1\text{H}^+$
^{78}Se	23.78	$^{38}\text{Ar}^{40}\text{Ar}^+$, $^{31}\text{P}_2^{16}\text{O}^+$
^{80}Se	49.61	$^{40}\text{Ar}_2^+$, $^1\text{H}^{79}\text{Br}^+$
^{82}Se	8.73	$^{12}\text{C}^{35}\text{Cl}_2^+$, $^{34}\text{S}^{16}\text{O}_3^+$, $^{82}\text{Kr}^+$, $^{40}\text{Ar}_2^1\text{H}_2^+$, $^1\text{H}^{81}\text{Br}^+$

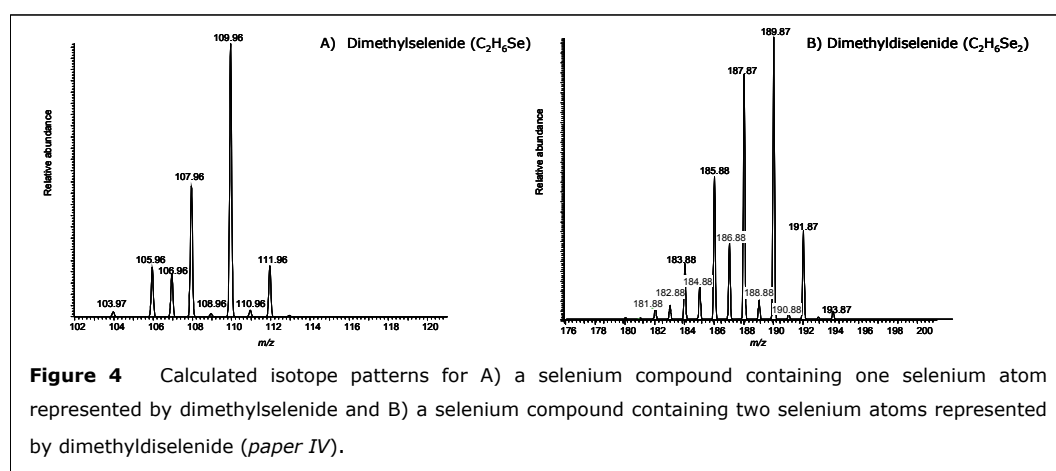
Although the sensitivity of selenium with argon ICP-MS is considered poor it is still very sensitive compared to ESI-MS. For most selenium compounds the sensitivity of ICP-MS is often 2-3 orders of magnitude higher than the sensitivity of electrospray ionisation (ESI)-MS[26]. Typical detection

limits for selenium in ICP-MS quadrupole instruments are 10-100 ppt. This is an average sensitivity as elemental detection limits covers the range of less than 1 ppt to more than 10 ppb. Only a few elements are not suitable for ICP-MS detection[32]. The sensitivity of selenium is influenced by the high 1st ionization potential leading to a degree of ionization of only 30%[33]. The sensitivity of selenium is also influenced by other matrix interferences that are not spectral interferences. These interferences may result in either enhancement or reduction of the selenium signal and they are greatly influenced by the operating conditions of the plasma[32]. Hence, selenium standards in the mobile phase were used for optimization of ICP-MS operating conditions.

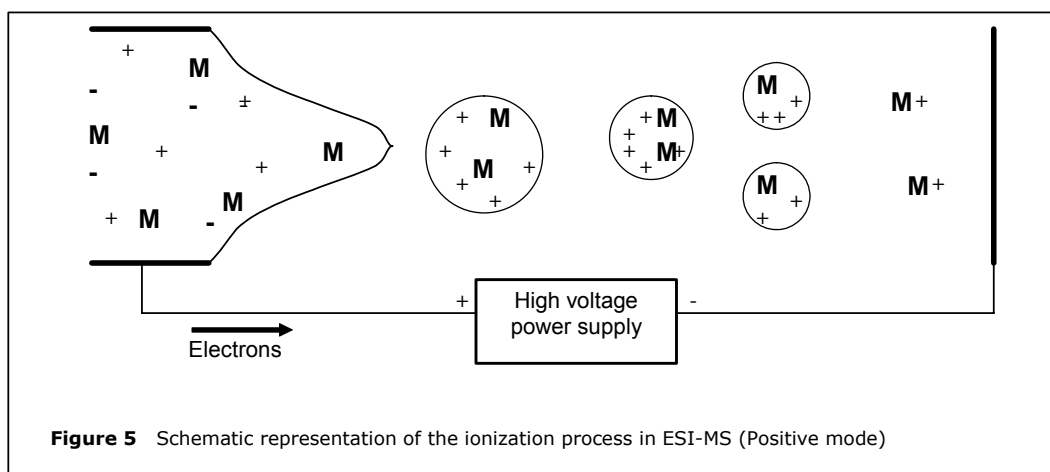
Sensitivity with respect to the detection limit of selenium was not an obstacle in this PhD project as the limit of detection for the ICP-MS detector was far lower than the limit of detection for the ESI-MS detector used for molecular identification. However, it is well known that selenium sensitivity can be enhanced by addition of carbon-containing solutes such as methanol to the mobile phase. Even small concentrations of organic solvent have a significant effect on selenium signal enhancement[34]. High concentrations of organic solvents however will extinguish the plasma. The amount of organic solvent entering the plasma can be reduced by cooling the spray chamber. Therefore, the spray chamber was kept at 5° C whenever methanol concentration above 5 % in the mobile phase was required for optimal chromatographic conditions.

2.1.2 ESI-MS (positive mode)

Molecular MS provides the structural information that is inherently lost in ICP-MS. Identification of selenium containing compounds by molecular MS is facilitated by the characteristic isotope pattern of selenium that is easily recognized. The characteristic isotope pattern for molecules containing one or two selenium atoms are shown in figure 4.



Since electrospray ionization is possible for a wide range of molecular masses and analyte polarities and it is relatively simple to use, it is often the ionization technique of choice in speciation analysis. An electrospray is produced by applying a strong electric field to a solution that is passed through a spray capillary. The electric field induces a charge accumulation at the surface of the solution located at the end of the capillary. The highly charged surface will break the solution into highly charged droplets i.e. an electrospray[35] (figure 5). The droplets are evaporated by a combination of heat and a stream of an inert drying gas, which causes them to shrink to the point where the repulsive power between the positively charged ions make the droplets break up into smaller droplets. This process will continue until at some point desorption of ions from the droplet surface occurs[35]. In principle the molecular ion of the analyte; M^+ is formed, however in the positive mode most often ions are formed by addition of a proton from the mobile phase, which results in protonated ions; $[M+H]^+$.



The softness of the ionization process generates ions in the gas phase which are very similar to the ions in the liquid phase as opposed to the ICP-MS ionization process. However collision with the drying gas may lead to a varying degree of fragmentation[29]. This so called insource fragmentation is relevant to minimize as it results in poorer sensitivity when the analyte signal is split up. The interface parameters are to be optimized to obtain complete desolvation of the ions without compromising their integrity.

One major disadvantage of the ESI technique is that it is highly prone to matrix interferences resulting in poor or lack of sensitivity to sample components of interest; to sustain the electrospray, electrochemical reactions take place in the tip of the spray capillary. As a consequence the total number of ions that can be extracted from the spray capillary is limited by the electric current produced by these electrochemical reactions, hence presence of interfering

compounds that are more prone to ionization may partly suppress the ionization efficiency of the analyte resulting in poor sensitivity. Furthermore, the electrospray droplets have to be completely desolvated in order for the ions to enter the mass spectrometer. The use of volatile solvents is therefore preferred and better sensitivity is often obtained with high concentrations of methanol or acetonitril that unfortunately are incompatible with ICP-MS.

3 Experimental

3.1 Instruments

LC-ICP-MS

The ICP-MS was a PE Sciex Elan 6000 (Perkin Elmer, Norwalk, CT, USA) equipped with a Micro Mist glass concentric nebulizer (Glass Expansion, Romainmontier, Switzerland) and a PC³ cyclonic spraychamber (Elemental Scientific Inc., Omaha, NE, USA). The sample uptake rate was 200 $\mu\text{L min}^{-1}$. ICP-MS sampler and skimmer cones were made of platinum. The plasma and auxiliary gas flow rates were 15 L min^{-1} and 1.2 L min^{-1} , respectively. The nebuliser gas flow, lens voltage and ICP RF power were optimized regularly with a solution of 100 $\mu\text{g Se L}^{-1}$ in mobile phase. The data requisition settings were: dwell-time 500 ms, sweeps per reading 1 and readings per replicate were varied corresponding to chromatographic runtime. ⁷⁷Se, ⁷⁸Se and ⁸²Se isotopes were monitored. The LC instrument was a G1376A capillary pump, a G1313A autosampler, a G1316A column compartment, a G1379A degasser and a G1314A variable wavelength detector, all from Agilent 1100 series, controlled by ChemStation software (Agilent Technologies, Waldbronn, Germany).

LC-ESI-MS (ion trap)

The ESI-MS ion trap detector was a G2445 LC/MSD Trap equipped with an API-electrospray interface (Agilent) controlled by LC/MSD Trap software (Bruker Daltronics Inc.) used for data acquisition and processing. The ESI-MS was coupled to a LC system consisting of a G1322A degasser, a G1312A binary pump, a G1315B diode array detector, a G1316A column compartment and a G1313A autosampler (all from Agilent). The electrospray was produced in the positive ionization mode. Details of the electrospray parameters are given in *papers I-III*.

LC-ESI-MS (triple quadrupole)

The ESI-MS triple quadrupole detector was a Thermo Finnigan TSQ Quantum Utra AM triple quadrupole mass spectrometer with a ESI interface coupled to a Thermo Finnigan Surveyor LC system (Thermo Fisher Scientific, Waltham, MA, USA). The electrospray was produced in the positive ionization mode. Details of the electrospray parameters are given in *paper III*.

3.2 Procedures

Unless otherwise stated the following procedures were applied.

Chromatography

All LC-ICP-MS analysis were performed with two Luna C18(2), 3 μ , 100Å, 2 mm ID \times 100 mm (Phenomenex, SupWare, Denmark) columns in series at ambient temperature unless otherwise stated in the figure legend. The flow rate was 200 $\mu\text{L min}^{-1}$. The mobile phase consisted of 200

mmol L⁻¹ ammoniumacetate in 5 % methanol unless otherwise stated in the figure legend. 12 µL sample aliquots were injected. UV detection was performed at 214 nm.

Digestion in simulated intestinal fluid (SIF)

SIF was freshly prepared and consisted of 10 mg ml⁻¹ pancreatin in 50 mmol L⁻¹ potassium phosphate buffer pH 6.8. Standards of SeMet, SeCys₂, Se-MeSeCys and selenite were digested at 37° C for 24 h under constant rotation. The concentration of selenium in the digestion samples were 1 mg L⁻¹. Digestion was terminated by protein precipitation with trichloroacetic acid in a final concentration of 2 %. The samples were filtered before subjected to LC-ICP-MS analysis. The standards were also incubated in the potassium phosphate buffer without enzyme.

In vitro digestion (IVD)

Selenium standards were incubated in pepsin 50 mg ml⁻¹ in 50 mmol L⁻¹ hydrochloric acid for 1 h at 37° C under constant stirring. This mimics gastric digestion. Hereafter pancreatin was added to a final concentration of 7.5 mg ml⁻¹ and sodium taurocholate was added to a final concentration of 5 mg ml⁻¹. Finally, pH was adjusted to approximately 6.5 with sodium hydrogencarbonate. The samples were further incubated at 37° C under constant stirring for 1 h. These conditions mimic the digestion in the proximal jejunum. After 1 h, sacks of tied off dialysis tube (cutoff Mw<5000) containing water was added to the digestion sample and digestion was allowed to proceed another 4 h under concurrent dialysis. Standards of SeMet, SeCys₂, Se-MeSeCys and selenite were digested at a final selenium concentration of 10 mg L⁻¹. The content of the dialysis sacks were analysed by LC-ICP-MS. For comparison the standards were incubated under similar conditions except that none of the enzymes pepsin and pancreatin were present.

In vitro intestinal metabolism

The small intestine from a pig was slit longitudinally and rinsed with ice-cold isotonic phosphate buffer (50 mmol L⁻¹ potassium dihydrogen phosphate and 90 mmol L⁻¹ sodium chloride adjusted to pH 7.4). The mucosal cells were scraped from the underlying muscle layers with a glass slide. The cells were suspended in isotonic phosphate buffer in a concentration of 20% w/w and homogenized with a Heidolph DIAX 600 homogenizer (VWR International, West Chester PA, USA) for 0.5 min. The homogenates were stored at -18° C. Standards of SeMet and Se-MeSeCys were incubated at 37° C for 24 h under constant rotation. The concentration of selenium in the incubation samples were 5 mg L⁻¹. Details of the incubation of selenite and MeSeA in intestinal epithelial cell homogenates are described in *paper I and II*, respectively. Incubation was terminated by protein precipitation with trichloroacetic acid in a final concentration of 2 % followed by centrifugation at 5300 rpm for 15 min. The supernatant was filtered through a 0.45 µm syringe cellulose filter before LC-ICP-MS analysis.

Hepatic metabolism (hepatocytes)

Hepatocytes were isolated from Sprague Dawley rats (150-200 g) obtained from Charles River Laboratories (Sulzfeld, Germany). The isolation was performed according to the two-step perfusion model described by LeCluyse *et al.* [36] and cryopreserved as described by Le Cam *et al.* [37]. Isolated hepatocytes were suspended in Dulbecco's modified Eagle's medium containing 10 % dimethyl sulfoxide. The suspension was immediately frozen at -20°C for 20 min followed by one hour storage at -80°C before storage in liquid nitrogen. Upon thawing, cryopreserved hepatocytes were suspended in Dulbecco's modified Eagle's medium. Cell viability was determined by the trypan blue exclusion method. At the end of the incubation periode, the cells were separated from the medium by centrifugation at 4000 g for 5 min. The incubation medium was analyzed directly, while the cell pellet was treated with acetonitrile. Acetonitrile extract was evaporated under a N₂ stream. The residue was sonicated after addition of a solution of 20 mmol L⁻¹ ammonium acetate in 2 % methanol. This fraction was separated by centrifugation 1000 g for 5 min and the supernatant was analyzed. The remaining pellet was solubilised in 5 % sodium dodecyl phosphate by sonification prior to total selenium analysis. All samples were stored at - 20°C until analysis. Total selenium analyses were performed by flow injection ⁸²Se-ICP-MS using the standard addition method.

4 Results and discussion

In this thesis the metabolism of selected selenium species were investigated in simulated gastrointestinal fluids, in an intestinal and in a hepatic model. Published results and unpublished results are presented and discussed collectively.

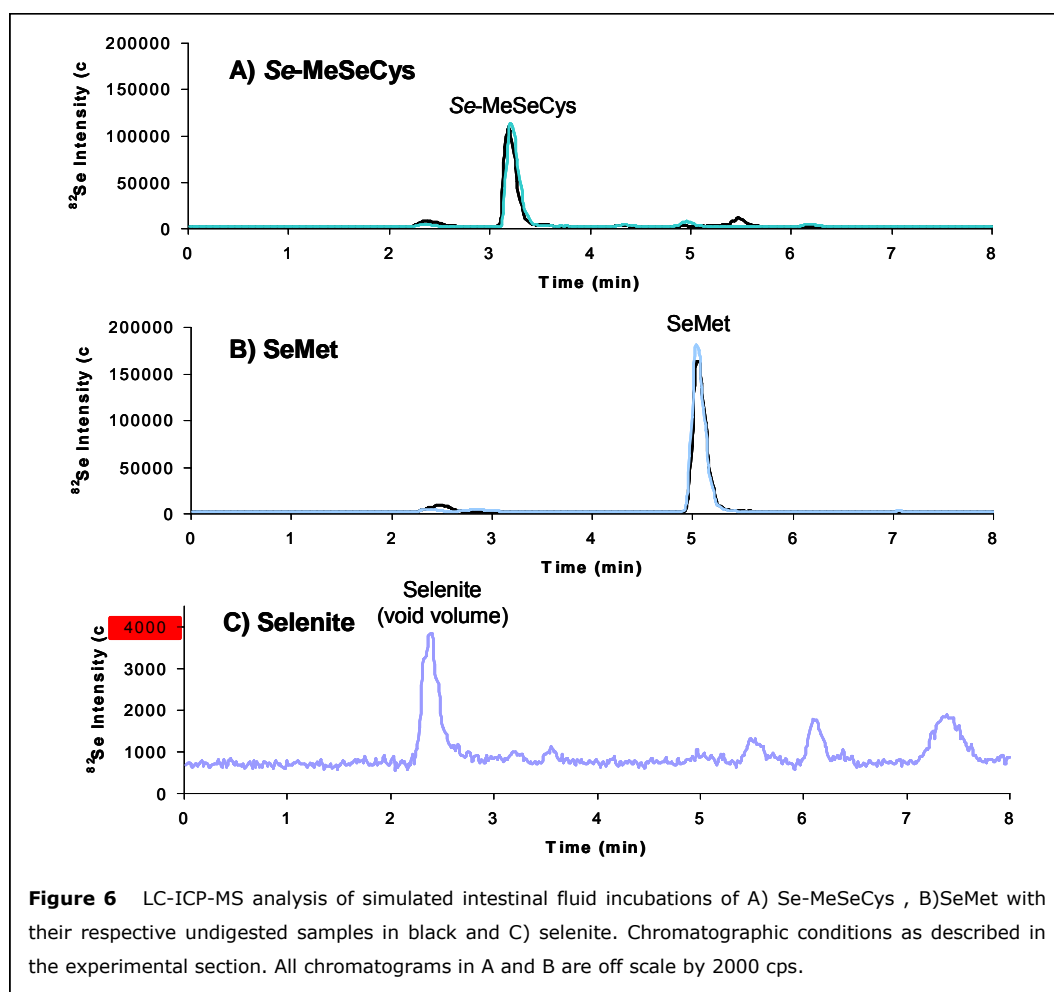
4.1 Gastro-intestinal digestion (unpublished results)

In order to examine if selected selenium compounds were changed during passage of the gastrointestinal tract, studies in simulated intestinal fluid[38] (SIF) were performed (results not published). SIF was freshly prepared and consisted of 10 mg/ml pancreatic enzyme in phosphate buffer pH 6.8. Standards of SeMet, SeCys₂, Se-MeSeCys and selenite were digested at 37° C for 24 h under constant rotation. Digestion was terminated by protein precipitation and the samples were filtered before subjected to LC-ICP-MS analysis. The standards were also incubated in the phosphate buffer without enzyme. SIF was prepared according to the US pharmacopoeia[38] in which it is primarily used for dissolution tests of enteric coated solid dosage forms. It contains the proteolytic enzyme pancreatin and has the salinity and pH of the duodenum and the proximal jejunum.

Se-MeSeCys was not stable in buffer which resulted in reduction of the Se-MeSeCys peak area in the LC-ICP-MS chromatograms. No additional digestion of Se-MeSeCys was observed when the enzyme was present (figure 6A). SeMet was stable in buffer and was not digested by the enzyme in SIF (figure 6B). Selenite was stable in the buffer but when subjected to SIF digestion selenium disappeared (figure 6C). Selenium from selenite was probably volatilised or associated to the insoluble protein fraction of the incubation sample. Results of repeated incubations of SeCys₂ were inconsistent, hence no reliable results was obtained for this species.

The selenium standards were also investigated in an *In vitro* digestion model (IVD), originally developed to investigate protein interactions with intestinal absorption of inorganic iron[39]. This model includes simulated gastric digestion for 1 hour before introduction of simulated intestinal digestion conditions for 4 hours. Digestion was followed by dialysis which allowed passing of small molecules. Standards of SeMet, Se-MeSeCys, SeCys₂ and selenite were incubated.

No digestion and no loss of selenium species was observed in the dialyzed samples compared to samples treated in the same way except that no enzymes were present during incubation. This is not in consistency with the results of SIF digestion; SeMet was not changed in any of the models but Se-MeSeCys and selenite underwent some transformation in SIF.



The SIF and IVD models are inherently rather similar. The models differ only in that the IVD model in addition to the pancreatic enzyme also contains a gastric enzyme and bile salts and they differ in incubation time. The different results obtained in the two models may be due to the different incubation time; In SIF standards was digested for 24 h and in the IVD model gastric digestion occurred for 1 hour followed by 5 h intestinal digestion. Impact of incubation time was not further investigated. However, it could be argued that long gastric digestion is irrelevant as the ventricle will be emptied in 4 hours after a meal is ingested. 7-8 hours post ingestion; indigestible remains of a meal have passed the intestine and reached the colon. In the colon very few species in general are able to be absorbed[40]. Hence, species that are not digested during 8 h may either be absorbed in their original form or excreted via faeces and thereby not systemic available.

Regarding the selenoamino acids our results are in concordance with the results of Dumont *et al*[41] who concurrently published similar studies in simulated gastric and intestinal juices. They also found that no further digestion of SeMet and Se-MeSeCys appeared to take place. They recovered 95-100 % of the dosed selenium of selenite by flow injection analysis of the digested sample which indicates that selenium of selenite is associated to the insoluble fraction. However Dumont *et al* did not report whether any digestion of selenite was observed. The main aim of the studies by Dumont *et al* was to assess which species were produced during gastrointestinal digestion of selenized yeast[41] and selenized garlic[42]. The main digestion products of selenized yeast were SeMet and SeCys₂. Digestion products of garlic were SeMet, Se-MeSeCys and γ -glutamyl-methylselenocysteine. However they reported that γ -glutamyl-Se-methylselenocysteine was extensively digested in the gastrointestinal fluids leaving Se-MeSeCys as the major digestion product of selenized garlic. Also Reyes *et al*[43] identified SeMet as a major digestion product of selenized yeast. However they also presented Se-containing peptides that was randomly produced by the gastrointestinal enzymes. It is very likely that these fragments of incompletely digested proteins contain selenium as selenomethionine. Whether these Se-containing peptides are relevant for absorption depends largely on their size or whether they are transported by peptide carriers in the gastrointestinal tract[44].

In conclusion, the digestion experiments showed that the selenoamino acids SeMet and Se-MeSeCys were not decomposed by the pancreatic and gastric enzymes, whereas selenite may be extensively decomposed into species that is not detectable by LC-ICP-MS analysis. Therefore no further digestion studies were performed. Based on the digestion studies it was concluded that SeMet, SeCys₂ and Se-MeSeCys were relevant model compounds for intestinal metabolism studies. Although it was not conclusive whether selenite was digested based on the two models, selenite was also subjected to intestinal metabolism studies.

4.2 Intestinal metabolism

Selenium metabolism was studied in homogenised pig intestinal epithelial cells as a model for intestinal metabolism. MeSeA was included as it has been widely used in cancer research as a model compound for methylated Se-amino acids, in particular Se-MeSeCys.

The results of the intestinal metabolism studies of selenite and MeSeA are published in *papers I and II*, respectively, whereas the results of incubations of SeMet and Se-MeSeCys are unpublished. For this model the intestine from anaesthetized pigs was removed, slit longitudinally and rinsed. The mucosal and epithelial cell layers were scraped of the underlying muscles layers, suspended and homogenised in saline phosphate buffer. The homogenates contains epithelial cells, their contents and intestinal mucosa. Selenium standards were added and incubated at 37°. Protein and cellular membrane residues were precipitated and the soluble fractions were analysed for

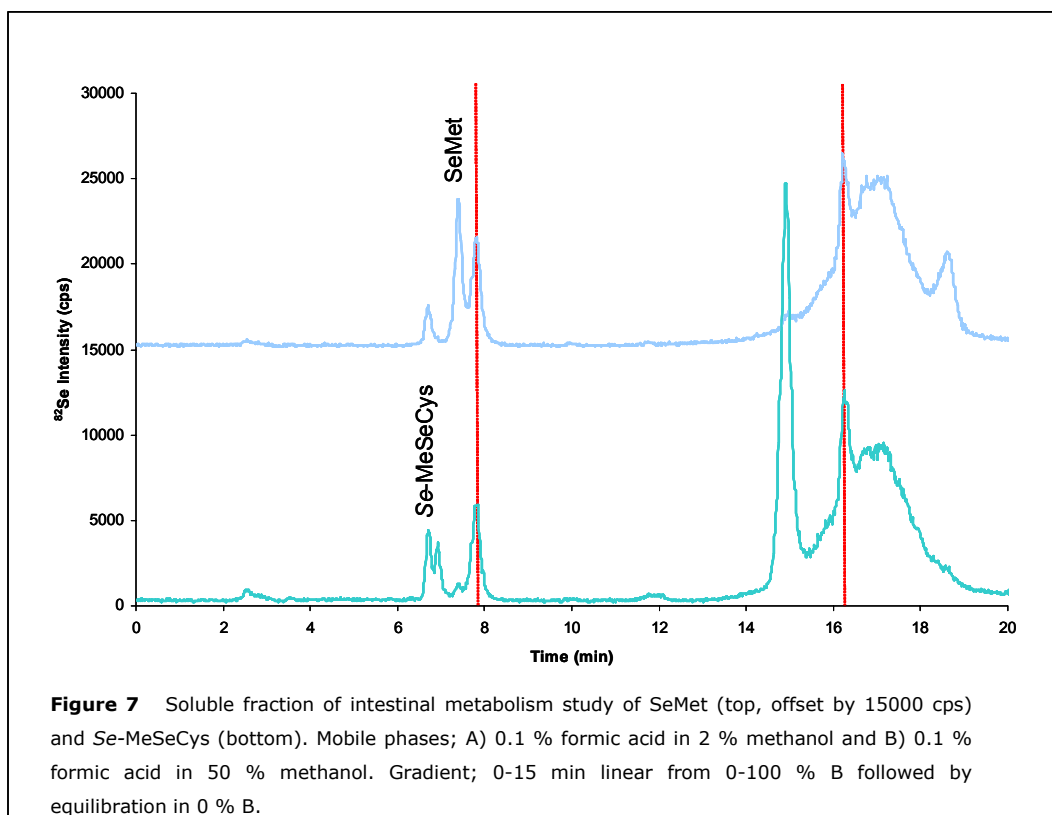
appearance of metabolites. The analysis for metabolites was limited to the soluble fraction, and did not include volatile or precipitated metabolites.

Epithelial cell homogenates like tissue homogenates provides data on intestinal metabolism pathways. However, extrapolation to *in vivo* conditions might be compromised by differences in model animal and human enzymes and their abundance[45,46]. As it was not known which enzyme pathways that would be involved in metabolism of the selenium compounds the epithelial cell homogenates were not standardized to a specific biological activity. It was found that the principal conversion of the selenium compounds was spontaneous and did not require enzymatic activity and therefore, the homogenates were not further characterized.

4.2.1 *Selenoamino acids (unpublished results)*

Extensive conversion of the selenoamino acids appeared as they were only recovered in limited amounts in the soluble fraction after incubation. However, only small amounts of metabolites of SeMet and Se-MeSeCys were observed in the soluble fraction (figure 7). Results from different epithelial cell homogenates from different pigs, differed quantitatively in the rate of disappearance and also to smaller extent qualitative differences were observed. The epithelial cell model is not well characterized and variation in the metabolic enzyme panel and enzyme amounts may account for different findings in different animals and epithelial cell homogenate batches. The epithelial cell homogenates may also be altered upon storage and this could cause different outcome of incubations too.

Although the metabolites of SeMet and Se-MeSeCys were not identified, LC-ICP-MS analyses of the respective samples indicate that the species may have at least two common metabolites observed at retention time 7.9 min and 16.5 min in the chromatograms. The broad peak eluting at 14-20 min is increased background due to increasing methanol content in the mobile phase. Several selenium containing compounds were strongly retained by the column; hence this experiment stresses the need for gradient elution with larger amounts of organic solvents, which make ICP-MS detection troublesome.

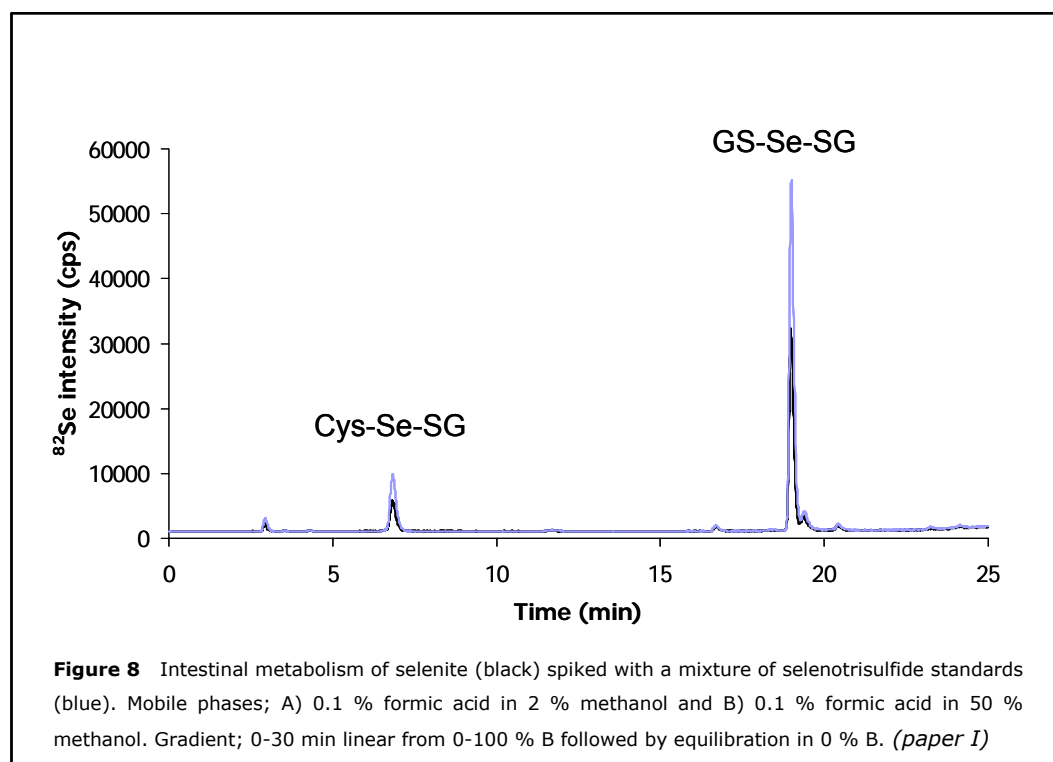


According to these results, SeMet and Se-MeSeCys may be metabolised by intestinal epithelial cells and delivered to the body in another form. However, the standards were incubated with the epithelial cell homogenates for 24 h, which is much longer than the intestinal transition time *in vivo*. No further experiments on SeMet and Se-MeSeCys intestinal metabolism were performed.

4.2.2 Selenite (paper I)

When selenite was incubated with homogenized epithelial cells, two major selenium containing compounds were observed in the soluble fraction. Minor selenium containing compounds were observed in the void volume (figure 8). This could be excess selenite or other hydrophilic compounds not retained by the column. The two compounds were formed instantaneously and they were very labile as the amount declined during a 15 min period. No other selenium containing peaks appeared instead. Hence, the secondary reaction products were volatile or precipitated. The primary compounds were stabilised by acidification which made them stable enough for further handling. The identity of the compounds was tentatively identified as the selenotrisulfide of glutathione (GSH) and the mixed selenotrisulfide of GSH (GS-Se-SG) and cysteine (GS-Se-Cys),

respectively as their retention times were identical when spiked with standards of the selenotrisulfides.

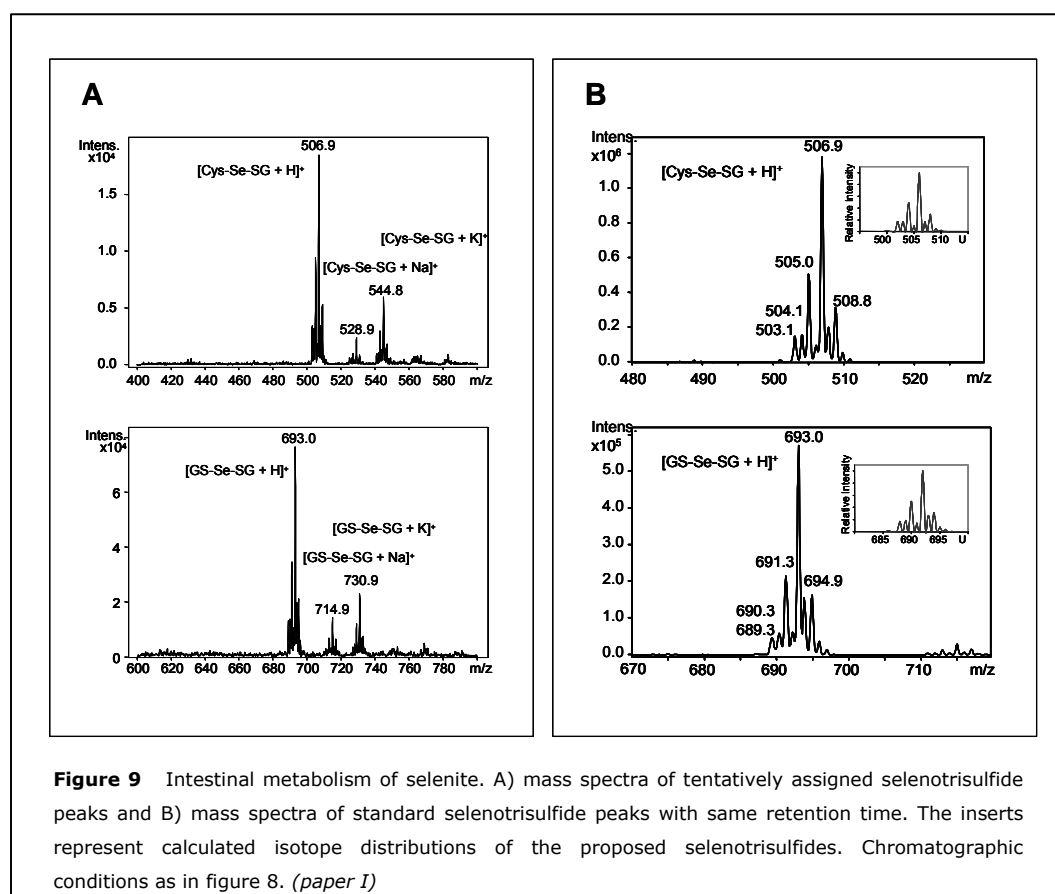


A selenium rich sample was prepared, in order to obtain the selenotrisulfides in concentrations suitable for detection by LC-ESI-MS. Based on retention time matching with the ordinary sample, the same selenotrisulfides were observed irrespective of the amount of selenite incubated. LC-ESI-MS spectra of the tentatively assigned selenotrisulfide peaks in the sample and LC-ESI-MS spectra of selenotrisulfide peaks in the standard were similar except for the presence of sodium and potassium adducts in the intestinal sample (figure 9).

Standards of the selenotrisulfides were prepared simply by mixing an aqueous solution of GSH and Cys with an acidified solution of selenite, hence formation of selenotrisulfides does not require any metabolic enzymes and they are formed instantaneously whenever selenite and GSH and/or Cys are present at the same time. Nevertheless, the selenotrisulfides are metabolically important as the thiols are ubiquitously present through out the gastrointestinal tract[47].

The formation of GS-Se-Cys and GS-Se-SG has never been identified by LC-ESI-MS in intestinal models before. Experiments of the absorption of selenite in models of rat intestine have shown that

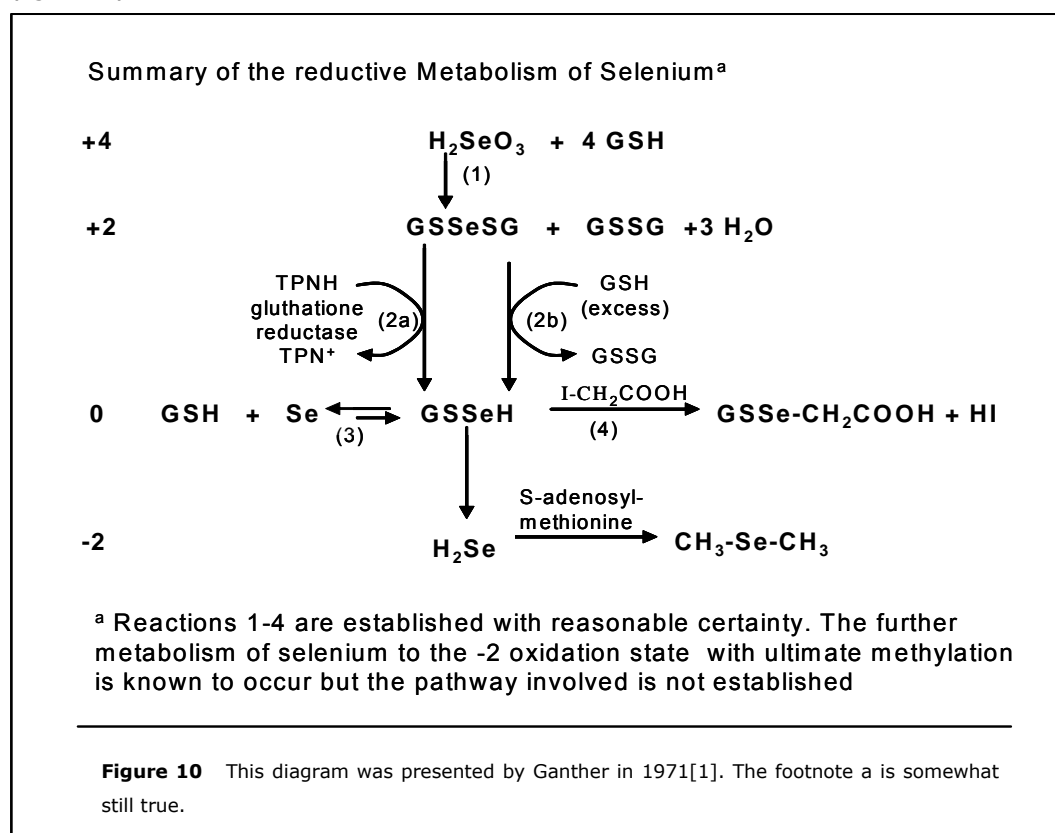
the presence of GSH increased cellular accumulation of ^{75}Se -labelled selenite[48] and that uptake of GS-Se-SG and Cys-Se-Cys was ten times faster than for selenium in the form of selenite[49]. The presence of selenite, GS-Se-SG, GS-Se-Cys, Cys-Se-Cys and protein-bound selenium in perfusates of the intestinal lumen of the rat has been proposed[50]. Identification of the selenotrisulfides in all of these experiments was based on unspecific ^{75}Se measurements or co-elution with standards in different chromatographic systems. Braga et al[51] showed formation of Cys-Se-Cys and GS-Se-SG in homogenized livers of rats injected intraperitoneal with selenite. The identification of the selenotrisulfides was based on co-elution with standards. The identity of the standards was established by LC-ESI-MS.



Selenotrisulfides of other biological relevant thiol compounds such as Coenzyme A[52,53], 2-mercaptoethanol[52], reduced ribonuclease A[54], homocysteine[51], dihydrolipoic acid[53], mercaptoethylamine[55] and cysteine[55] have been synthesized in aqueous solution. Some for reaction mechanism studies and others as standards for chromatographic retention time

comparison and mass spectrometry. The selenotrisulfides of mercaptoethylamine[55] and cysteine[51,55,56] have been detected in biological samples. However their physiological relevance has not been elucidated. Our investigation of selenite metabolism in intestinal epithelial cell homogenates indicates that besides GS-Se-SG also substantial amounts of the mixed selenotrisulfide Cys-Se-SG most certainly are formed *in vivo*. Hence, besides investigations of GS-Se-SG, further research in selenite absorption and metabolism pathways must include other selenotrisulfides, at least the selenotrisulfides of cysteine; Cys-Se-SG and Cys-Se-Cys.

Selenotrisulfides are well known reaction products of selenite and thiol compounds and during the last century this reaction was thoroughly investigated for stoichiometry and reaction mechanism. Still, questions about the reaction in relation to physiology are unresolved. Painter reviewed selenium chemistry and toxicity in 1941 and reported the reduction of seleninic acid by thiol compounds[57]. In the late 1960's and early 1970's Ganther published several papers on selenotrisulfide studies suggesting the series of reduction reactions most referred to hereafter (figure 10) [1,52,58].



With enzymatic techniques, Kramer and Ames[59] further integrated formation of the superoxide anion in the reaction scheme. At the same time Vendeland *et al* published molecular mass spectra of GS-Se-SG and Cys-Se-Cys in aqueous solution[49] and later Braga *et al* published mass spectra of Cys-Se-SG and the selenotrisulfide of homocysteine (HCys-Se-HCys) standards[51].

The reductive metabolism of selenite produces intermediate species i.e. HSe^- and perselenides (R-SeH), that are highly reactive or volatile, hence positive identification of all involved species imposes serious analytical challenges. Spontaneous formation of the proposed intermediate perselenide (GS-SeH) was shown by Ganther[1] as the reaction product of iodoacetate. The identification of the derivative (GS-Se-acetate) was based on retention time shifts in electrophoretic and chromatographic systems. However GS-SeH has never been isolated and identified as such, probably because it is far too reactive to be stable in solution. Formation of the volatilized HSe^- has been concluded by loss of selenium from reaction mixtures upon acidification. The relative quantity of HSe^- formation has not been reported. Formation of elemental selenium is reported based on visual observation of red precipitate in reaction mixtures or by measuring the absorbance at wavelength 400 nm. Today this kind of analytical evidence is considered obsolete. Nevertheless, despite great technical advances the quote "The further metabolism of selenium in the -2 oxidation state with ultimate methylation is known to occur but the pathway involved is not established" of Ganther from 1971 is still somewhat true.

The formation of GS-Se-SG is well established and the compound has been identified by molecular MS in aqueous standards[49,51], in aqueous yeast extracts[56] and now in intestinal epithelial cell homogenates. The LC-ICP-MS analysis of the aqueous GS-Se-SG standard revealed an additional minor peak (figure 11). LC-ESI-MS analysis revealed a spectrum with the characteristic isotope pattern of a compound containing two selenium atoms at m/z 772.4 for the $^{80}\text{Se}^{80}\text{Se}$ compound (figure 11). The spectrum is similar to the theoretically calculated isotope pattern of diselenodiglutathione (GS-Se-Se-SG). This compound has never been reported as part of the reductive reaction cascade of selenite and GSH (*unpublished results*).

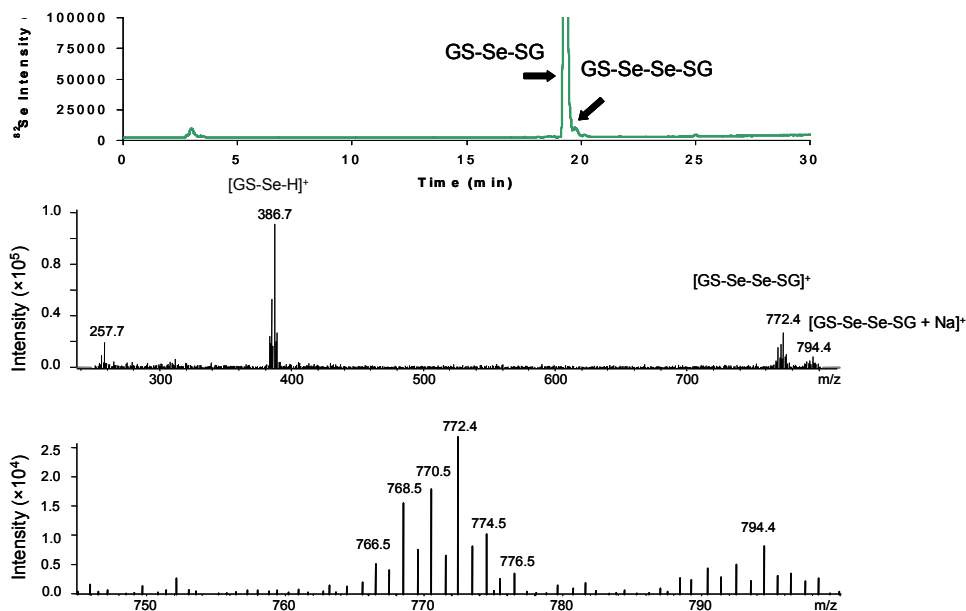


Figure 11 LC-ICP-MS and LC-ESI-MS analysis of an aqueous mixture of GSH and selenite. Chromatographic conditions as in figure 8.

As glutathione is widely present throughout the animal and human bodies the most important selenotrisulfide is considered to be selenodiglutathione (GS-Se-SG). GS-Se-SG is essentially unstable at physiological conditions i.e. pH approximating neutral and GSH excess. In the stomach, the selenotrisulfides may be stable because the pH is low, but in the intestine where pH reaches neutral, the selenotrisulfides are most certainly unstable. The *in vivo* fate of the selenium from the rapid decomposition of the selenotrisulfides has been widely speculated and discussed, but no clear evidence for a specific metabolic pathway has been provided. Ganther proposed that in addition to the spontaneous reduction of selenite by GSH the reduction was enhanced by action of the enzyme Glutathione Reductase (GR) to yield diglutathione (GS-SG) and HSe^- (figure 12). GR is a ubiquitously present enzyme that reduces diglutathione to glutathione using redox equivalents from NADPH[1].

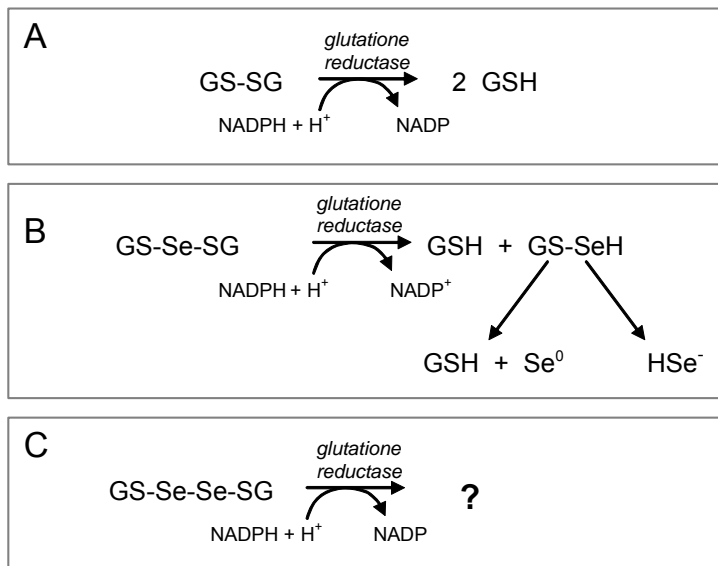


Figure 12 Reactions mediated by glutathione reductase (GR)[1]. Reaction C has never been investigated and it is highly speculative.

The reactive and toxic intermediate HSe^- is often referred to as the key intermediate in selenium metabolism available for selenium incorporation into proteins and for excretion via methylation. In biological material, formation of red elemental selenium ($\text{Se}(0)$) has not been reported suggesting existence of biological mechanisms that prevent the formation of $\text{Se}(0)$. Rapid methylation or incorporation into selenoproteins via selenophosphate may be one mechanism. Another proposed fate of the selenotrisulfides is that selenium is directly donated to sulfhydryl groups of proteins. The rationale for this mechanism would be that the cell avoids free HSe^- which is highly toxic[60]. Incorporation of selenium into selenoproteins takes place via a specific translational codon for selenocysteinyl-tRNA. Selenocysteinyl-tRNA is synthesized via selenophosphate. Selenophosphate is synthesized by selenophosphate synthetase (SPS) as shown in figure 13. SPS is claimed to have HSe^- as the selenium donating substrate and adenosinetriphosphate (ATP) as the phosphate donating substrate[61,62].

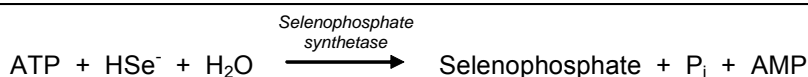


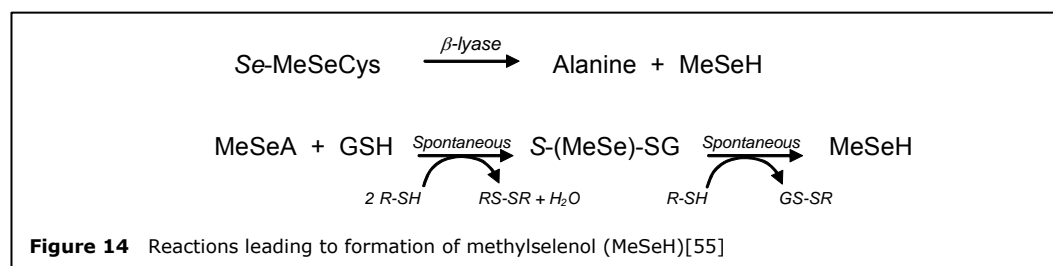
Figure 13 Reaction mechanism of selenophosphate synthetase (SPS)[49]

HSe⁻ is claimed to be produced *in situ* by the reduction of selenite. Whether selenite is reduced to HSe⁻ or it is the intermediate reduction products that are substrates for selenophosphate synthetase has not been proven; however the reduction of selenite is pre-requisite for any incorporation of selenite selenium into selenophosphate[62,63].

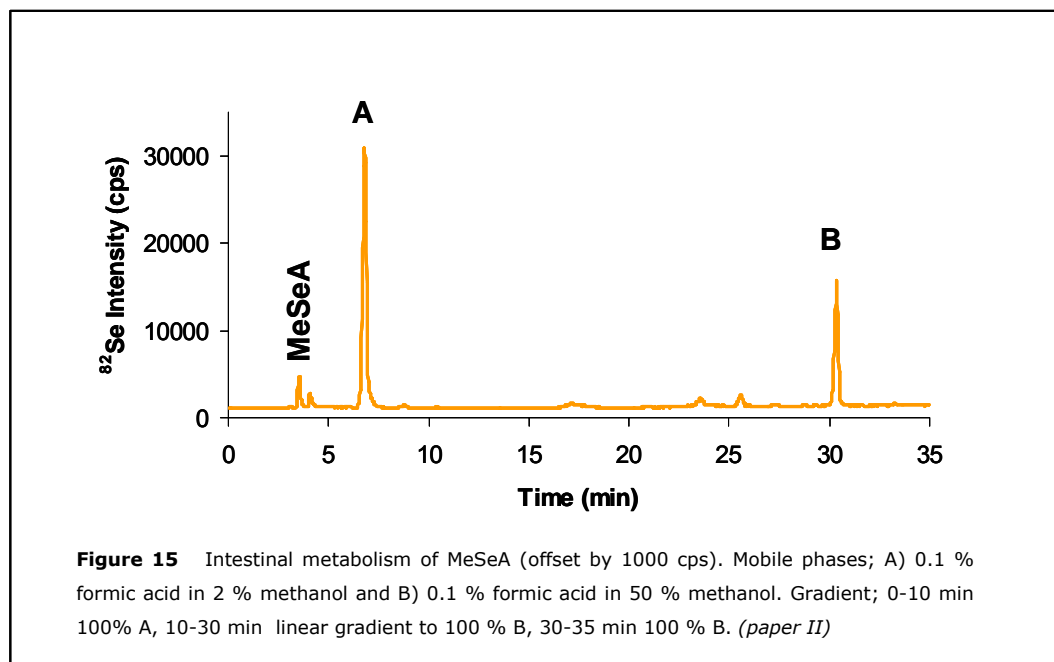
In conclusion, although our *in vitro* intestinal metabolism studies of selenite have not added any further evidence for pathways of selenite metabolism, they emphasize the fact that selenite is reduced intestinally and will never be bioavailable in its intact form. What is really missing is the identity of the form in which selenium of selenite origin is absorbed from the intestine.

4.2.3 Methylseleninic acid (paper II)

MeSeA was first introduced as a model compound for selenoamino acids by Ip *et al* [64] in 2000 and as it is not naturally occurring it had gained limited interest in metabolism research until then. Se-MeSeCys is believed to produce MeSeH when cleaved by the β -lyase enzymes and MeSeA is believed to produce MeSeH by a series of spontaneous reductions by thiols such as Cys and GSH[2] (figure 14). Hence, MeSeA has been widely used in cancer research as a precursor for MeSeH in studies performed both *in vitro* and *in vivo* to ensure formation of MeSeH despite low β -lyase activity in target tissue or *in vitro* models[2,64-68].



In our studies, MeSeA was extensively metabolised when incubated with homogenized epithelial cells and one major selenium containing compound (**A**) was observed in the soluble fraction. The compound was formed immediately after addition of MeSeA to the cell homogenate. In contrary to the selenotrisulfides identified when selenite was added to the cell homogenate, this compound remained present in the soluble fraction even after 24 h incubation. However, not all of the selenium applied to the column could be accounted for and analysis of the sample by use of gradient elution revealed another selenium containing compound (**B**) in the soluble fraction (figure 15). The size of this peak varied in between incubations. In addition, the relative peak sizes were not comparable as the ICP-MS sensitivity is greatly influenced by the changing methanol content of the mobile phase in gradient elution, making quantitation of the compounds difficult.



The sample was subjected to LC-ESI-MS, but no peaks with a spectrum with the characteristic selenium isotope pattern were observed at the retention times of the unknown compounds. Following, the sample was purified by preparative chromatography in two different mobile phases. The mobile phases contained 0.1% formic acid (pH 2.6) and 0.1% ammonium formate (pH 7), respectively. The fractionation on the preparative column was monitored by splitting the flow and continuously monitoring the selenium output by ICP-MS in order to collect the relevant fraction. The retention time of the metabolite did not change in the different systems, but from the UV-trace (not shown) it appeared that the purity of the metabolite increased by separating the metabolite from different impurities in the two systems. The purified sample was analysed by LC-ESI-MS and a peak with a spectrum containing the characteristic selenium isotope pattern was observed at the retention time of the major selenium containing metabolite (A)(figure 16). Purification and pre-concentration of the other selenium containing metabolite (B) was not successful.

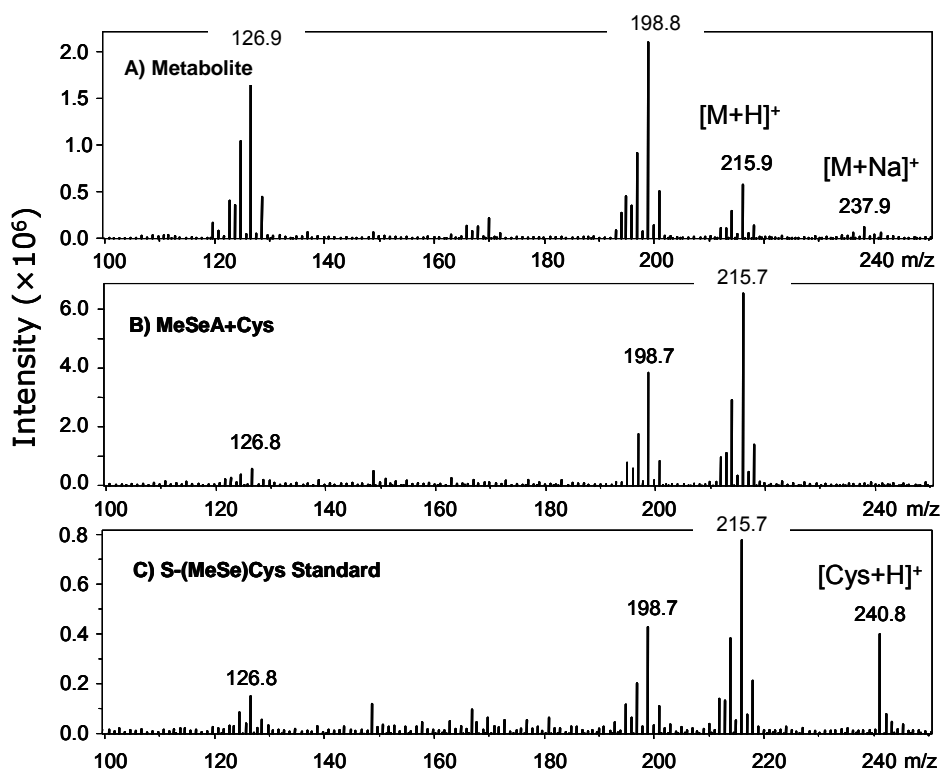
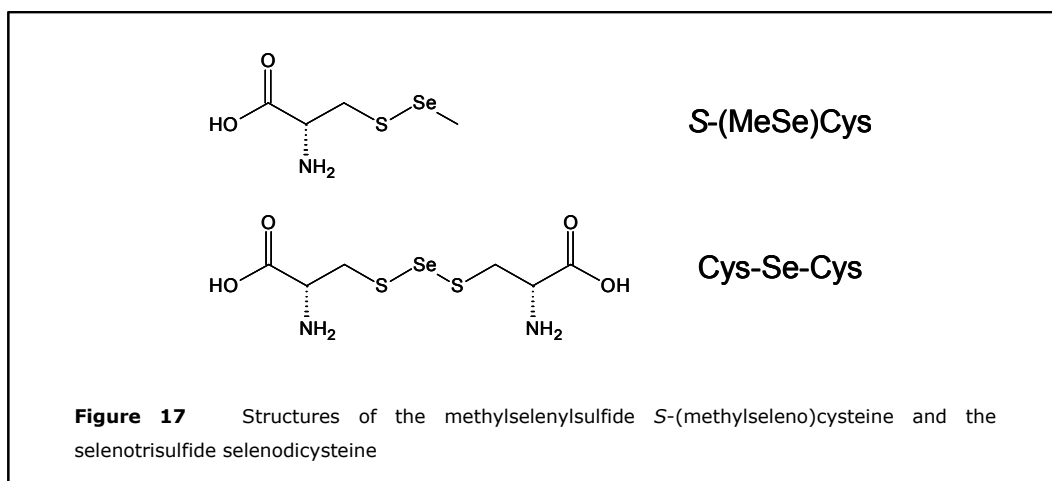


Figure 16 LC-ESI-MS spectra of A) purified and pre-concentrated metabolite of MeSeA, B) aqueous mixture of GSH and MeSeA and C) synthesised S-(MeSe)Cys verified by NMR. The ion at m/z 240.8 is due to unresolved cysteine impurity also observed by NMR. (*paper III*)

The characteristic isotope pattern of selenium was identified at m/z 126.9, m/z 198.8 and m/z 215.9 corresponding to ^{80}Se . The ion at m/z 215.9 $[\text{CH}_3\text{SeCys}+\text{H}]^+$ is the parent ion, but due to excessive in-source fragmentation, the ion at m/z 198.8 is the most intense. A small signal with a selenium pattern was identified at m/z 237.9, which corresponds to the sodium adduct of m/z 215.9. Full scan spectra were recorded in the m/z range 150-800 to examine if the ion was a fragment ion, produced by in source fragmentation of a larger ion. However, no selenium isotope pattern was observed above m/z 250. Based on these data, the identity of the metabolite was expected to be the sulfur-selenium amino acid S-(methylseleno)cysteine (S-(MeSe)Cys). As this compound resembled the selenotrisulfide of cysteine (Cys-Se-Cys) (figure 17) except that selenium only coordinates with one sulphur, while the methylseleno group is preserved, it was investigated whether addition of an aqueous solution of MeSeA to Cys would produce the metabolite.



LC-ESI-MS spectra of the reaction product between MeSeA and Cys, a synthesised *S*-(MeSe)Cys standard verified by NMR and the unknown metabolite from the incubation sample, were similar (figure 16). Hence, the identity of the metabolite was finally verified and it was clear that the metabolite is formed spontaneously upon reaction of MeSeA and Cys present in the intestinal epithelial cell homogenates.

The other selenium containing compound observed in the soluble fraction of the epithelial cell homogenates, was tentatively identified by coelution when spiked with the reaction product of MeSeA and GSH. The reaction product of GSH and MeSeA was identified by LC-ESI-MSⁿ as *S*-(methylseleno)glutathione (*S*-(MeSe)SG).

S-(MeSe)Cys has not been identified in mammal models before. It was first detected in yeast by the Uden group, who proposed the presence of a selenium-sulphur compound in selenized yeast after proteolytic digestion, derivatisation and analysis by GC-MS[69]. The identity of the compound was following verified by ⁷⁷Se- and ¹H-NMR after synthesis of a standard and it was proposed that the selenylsulfides are intermediates in the catalytic cycle of glutathione peroxidase[70]. It is proved now, however, that *S*-(MeSe)Cys is a product of spontaneous reduction of MeSeA by Cys as well.

In conclusion, our studies showed that MeSeA like selenite was reduced by thiols Cys and GSH. Hence, MeSeA and selenite metabolism had more resemblance than the metabolism of MeSeA and SeMet, Se-MeSeCys or SeCys₂. This finding questions the relevance of the use of MeSeA as a model compound for methylated Se-amino acids in cancer research. The *in vitro* intestinal metabolism studies show that SeMet and Se-MeSeCys either is taken up by epithelial cells in their

intact form or is metabolised to give either volatile selenium or selenium species associated to the insoluble fraction. Selenite and MeSeA however, are completely reduced when administered orally and will not be available for absorption in their intact forms.

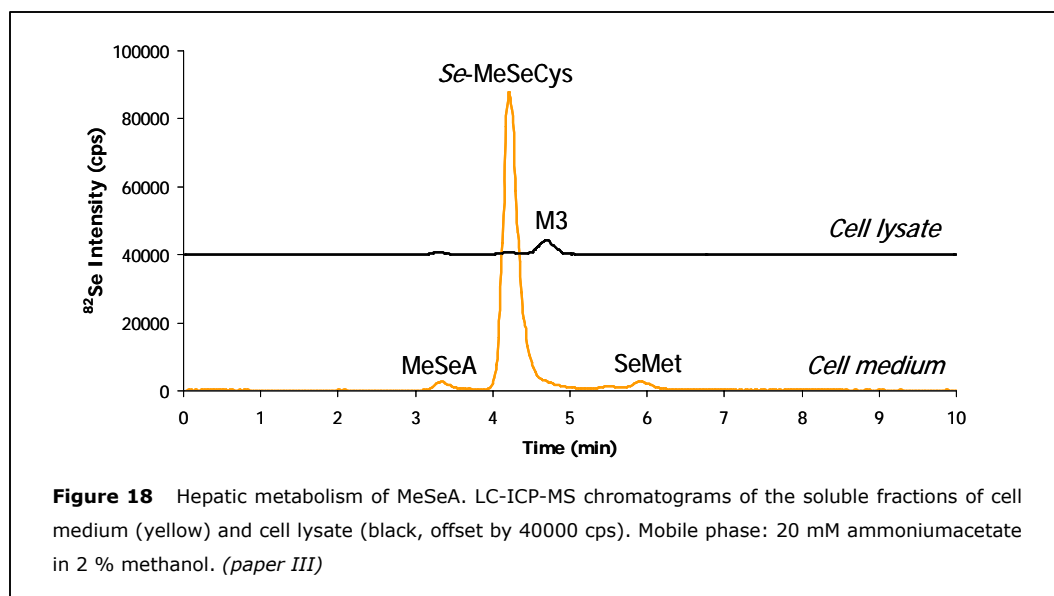
4.3 Hepatic metabolism

The liver is the primary organ involved in the metabolism of xenobiotics and many compounds are taken up by hepatocytes and converted to pharmacologically inactive, active or toxic metabolites. Before entering the systemic circulation, absorbed species are directed via the portal vein to the liver. Hence, first pass hepatic metabolism plays a major role in bioavailability of ingested compounds. As hepatocytes contain the full complement of enzymes and cofactors that a compound is likely to encounter during first passage of the liver, they are physiologically relevant for *in vitro* metabolism studies[71].

4.3.1 Methylseleninic acid (paper III)

Cryopreserved rat hepatocytes were incubated with MeSeA and the insoluble fractions were analysed by a combination of LC-ICP-MS and LC-ESI-MSⁿ experiments. The experiments and the results are submitted for publication in *paper III*. In cell medium, two metabolites (M1 and M2) were detected by LC-ICP-MS analysis. In the soluble fraction of methanolic cell lysates, two metabolites were detected (M1 and M3) (figure 18). The identities of metabolites M1 and M2 were tentatively established by chromatographic coelution with Se-MeSeCys and SeMet standards. It is important to notice that only about 15 % of the dosed selenium was recovered in the cell medium and even less was found in the soluble fraction of cell lysates (< 1 %). In fact, only about 25 % of the dosed selenium was accounted for by total selenium analysis of the medium, lysates and insoluble fraction. This indicates that about 75 % of the dosed selenium was lost, probably due to formation of volatile species. Infante *et al*[72] detected volatile selenium species dimethyldiselenide and dimethylselenide in the head space of lymphoma B-cells incubated with MeSeA, although no quantities were reported. Hence, formation of volatile selenium species by the hepatocytes is plausible. Preliminary experiments in which hepatocytes were incubated with MeSeA in the cell of a membrane inlet mass spectrometer (MIMS) with and electron impact (EI) ionisation source showed that large amounts of dimethyldiselenide was volatilised instantaneously from the incubation sample (*results not published*).

The hepatocytes were incubated with a large amount of MeSeA in order to obtain metabolites in a concentration allowing identification by molecular mass spectrometry. The dose was far above any physiological relevance. However, the metabolic pattern was qualitatively similar to that obtained with relevant doses of MeSeA. Additionally, viability of hepatocytes was not significantly altered upon treatment with the high dose of MeSeA.



Identification of M1 – Se-MeSeCys

LC-ESI-MS analysis of the purified and pre-concentrated metabolite M1 resulted in a spectrum with the characteristic selenium isotope pattern at m/z 184 corresponding to ^{80}Se at the retention time of Se-MeSeCys. Isolation and fragmentation of the parent ion (m/z 184) led to formation of an intense ion at m/z 167, corresponding to loss of ammonia ($[\text{M}-\text{NH}_3]^+$). This ion was isolated and further fragmented in a MS^3 experiment that resulted in fragment ions at m/z 149 $[(\text{M}-\text{NH}_3)-\text{H}_2\text{O}]^+$, 139 $[(\text{M}-\text{NH}_3)-\text{CO}]^+$, 123 $[(\text{M}-\text{NH}_3)-\text{COO}]^+$ and 95 $[\text{CH}_3\text{Se}]^+$ (figure 19). This fragmentation pattern was similar to the fragmentation pattern of a Se-MeSeCys standard. Similar MS^2 and MS^3 experiments of parent ion m/z 182 for the ^{78}Se compound also verified the extracted full scan selenium isotope pattern (table 2).

Table 2 LC-ESI- MS^n data Se -MeSeCys

	Fragment ions (m/z)	
	Standard	Sample
MS^2		
Isolated mass 184	166.9	166.9
Isolated mass 182	164.9	164.9
MS^3		
Isolated Masses 184/167	149.0	148.9
	138.9	138.9
	122.9	122.8
	95.0	95.1
Isolated Masses 182/165	-	146.9
	-	137.0
	-	121.0
	-	93.2

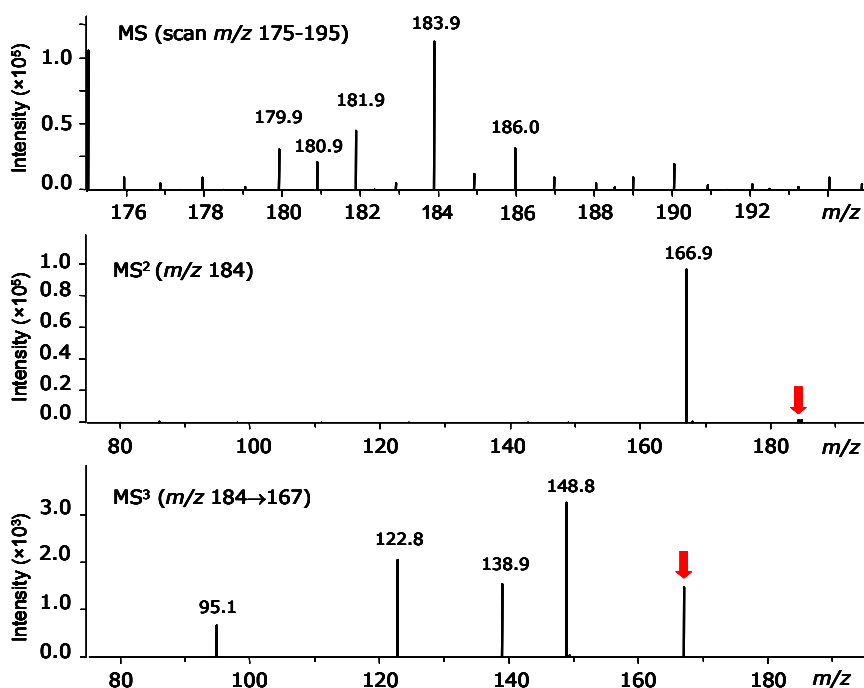


Figure 19 LC-ESI-MSⁿ spectra at retention time of Se-MeSeCys in purified and pre-concentrated metabolite M1. Mobile phase: 0.1 % formic acid in 2 % methanol. (*paper III*)

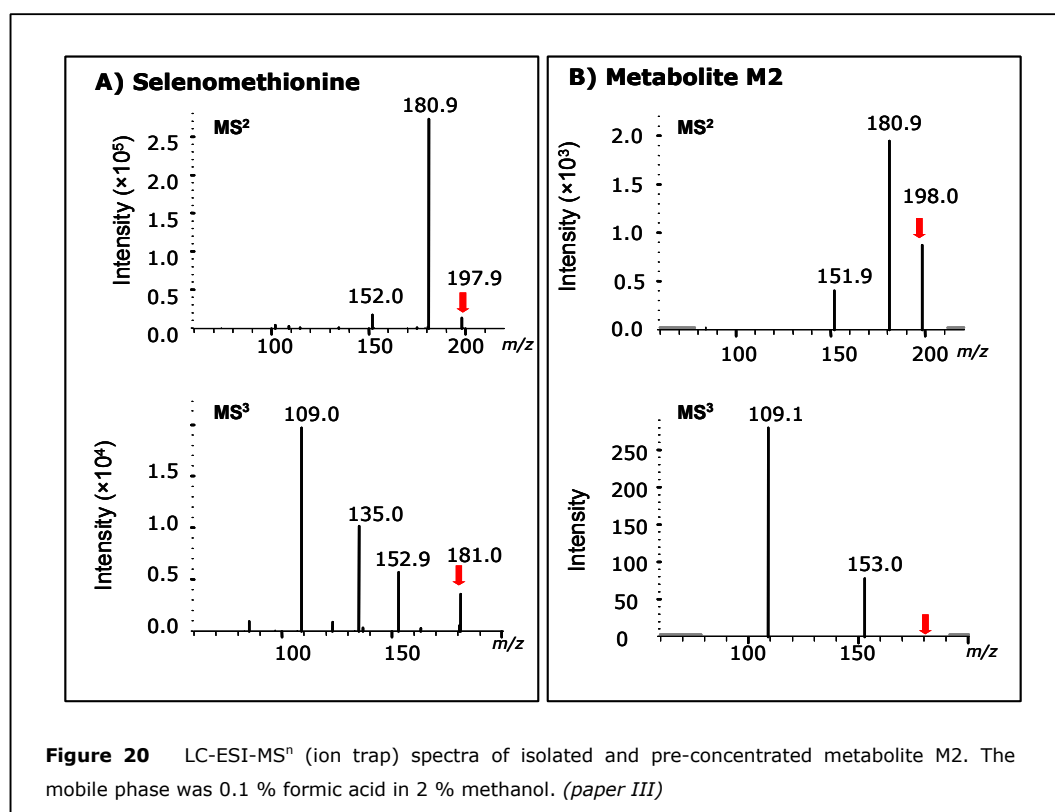
The major metabolite excreted from the hepatocytes upon incubation with MeSeA was Se-MeSeCys. Infante *et al* also identified Se-MeSeCys as a metabolite of MeSeA in the lymphoma B-cell line[72]. Hence, the capacity to produce Se-MeSeCys from MeSeA is not restricted to the very metabolically active cancer cells but is also present in normal healthy hepatocytes.

Identification of M2 - SeMet

Due to limited formation of metabolite M2 and limited amounts of incubation medium, the identity of this metabolite was established by two different molecular mass spectrometry approaches.

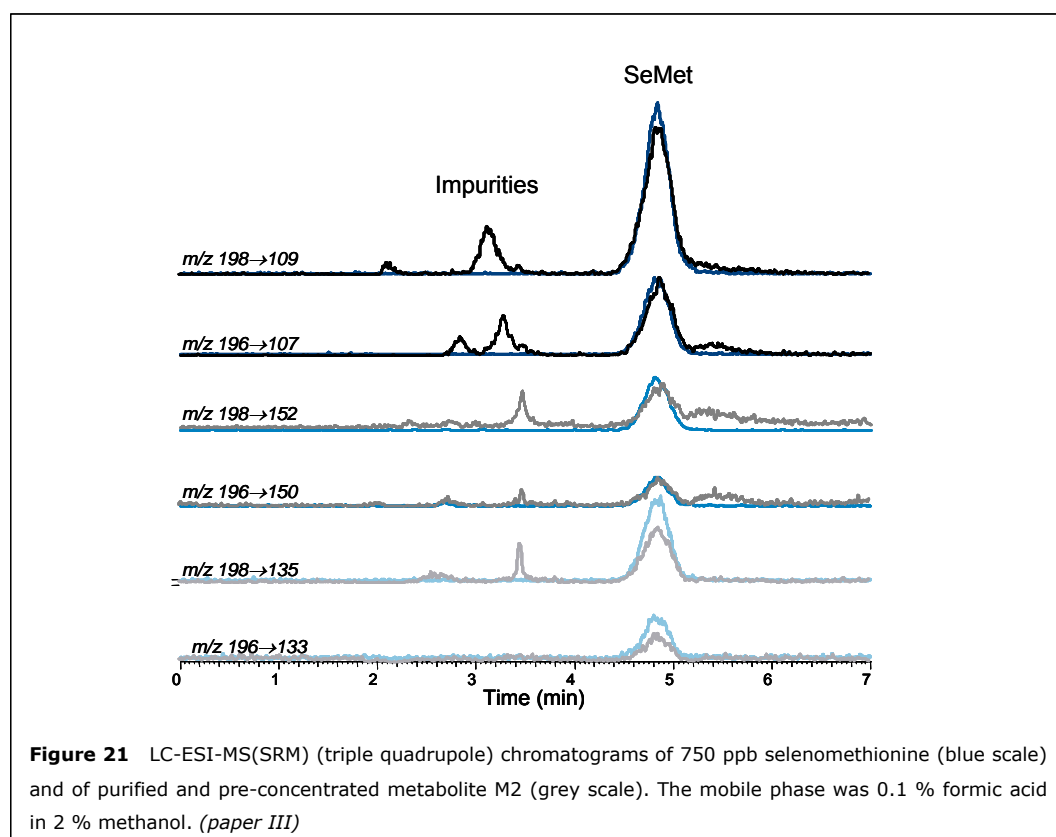
Full scan LC-ESI-MS² and LC-ESI-MS³ experiments were performed by use of an ion-trap instrument. Spectra in MS² mode of a SeMet standard and the purified and concentrated metabolite M2 were similar (figure 20). Isolation and fragmentation of the parent ion (m/z 198.0) led to formation of an intense ion at m/z 180.9, corresponding to the loss of ammonia ($[M-NH_3]^+$). A

minor fragment ion was observed at m/z 151.9 corresponding to parallel loss of formic acid ($[M-HCOOH]^+$). The major fragment ion (m/z 180.9) was isolated and further fragmented by LC-ESI-MS³ analyses. In the MS³ mode, the extracted spectrum for M2 showed two fragment ions at m/z 153.0 ($[(M-NH_3)-CO]^+$) and 109.1, corresponding to cleavage of the bond in α position to the Se atom ($[CH_3-Se-CH_2]^+$). The fragment ion at m/z 135.0 ($[(M-NH_3)-HCOOH]^+$), observed with SeMet standard, did not appear. The signal for the standard was approximately 10^4 times more intense than the signals for the metabolite. The low intensity for the sample was due to low concentration of the metabolite and probably also due to ion suppression in the biological sample. Although preparative chromatography was performed, it still contained salt and other interfering species. In this LC-ESI-MSⁿ experiment no peaks were observed in the total ion current (TIC) MS² and MS³ chromatograms at the expected retention time of SeMet. However, from the LC-ICP-MS analysis it was evident that the compound was present in the sample.



The LC-ESI-MSⁿ experiments were not considered solid proof of metabolite identity and the data was supplemented with data from another LC-ESI-MS instrument with a triple quadrupole mass analyzer (QQQ). Based on LC-ESI-MS² full scan spectra of a SeMet standard, three specific

transitions from the parent ion to the fragment ions (obtained by collision induced dissociation) were selected for selected reaction monitoring (SRM) mode analysis. Figure 21 shows the SRM chromatograms of the selected transitions for the purified and pre-concentrated metabolite M2 and a SeMet standard of similar concentration. In all SRM experiments, transitions for parent ion corresponding to both ^{80}Se and ^{78}Se were monitored. The chromatographic peak at the retention time of SeMet was observed for all selected transitions. Although the same chromatographic setup (identical column and mobile phase) was used in the two LC-ESI-MS instruments, the retention time of SeMet was slightly displaced due to different void volumes of the two instruments. SRM is considered a rather selective detection method. However, the purified and pre-concentrated sample contained species that exhibited one or more SRM transitions selected for identification of SeMet. In this case specificity is ensured by chromatographic separation of the species and the relative intensity of the transitions is compared to that of a standard (figure 21).



The main fragment produced in the triple quadrupole instrument was $m/z\ 109.0$, whereas the main fragment produced in the ion trap (MS^2 mode) was $m/z\ 181.0$. The $m/z\ 109.0$ fragment ion was

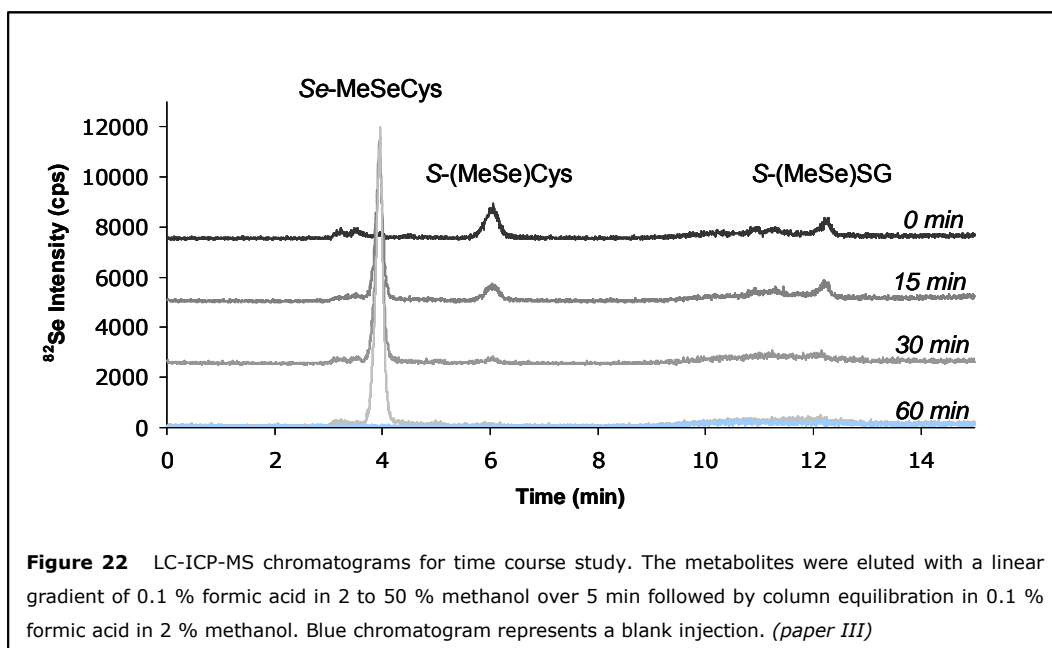
only observed in the MS³ mode on the ion trap instrument. As these significant different observations were done for both the unknown species M2 and the SeMet standard, the evidence of the identity of the species is improved.

The major drawback of molecular MS for identification of compounds is the need for reference spectra i.e. standard material must be available. Only limited structural information is available from MS for example typical losses identifying structural groups i.e loss of a methyl group (15 mass units) and ammonia (17 mass units) may be identified but the position of the groups is not given. To circumvent the need for reference material NMR is the analysis of choice. However, samples for NMR analysis has to be very clean, which is often a major obstacle in bioanalysis. In this case SeMet and Se-MeSeCys standards were well characterized and commercial available and the identity of metabolites M1 and M2 were established.

The lymphoma B-cells incubated with MeSeA by Infante *et al*[72] also produced SeMet. This observation was very surprising as it traditionally has been accepted that animals (including humans) were not capable of synthesizing SeMet but exclusively obtained it via food or nutritional supplements. Although MeSeA is not found in food and therefore not a part of the human selenium intake, the observation opens the question whether SeMet might be biosynthesized from other nutritionally available selenium compounds as well.

A short time course study with low dose of MeSeA was performed. As all MeSeA was instantaneously metabolized it was not possible to determine any reaction kinetics from this study (figure 22). Formation of Se-MeSeCys was observed after 15 min and the amount increased over the one hour duration of the study. SeMet was not detected. Another selenium containing compound was detected. The peak decreased in size over time and it was not detected after 60 min. A selenium containing compound was detected at limit of detection levels.

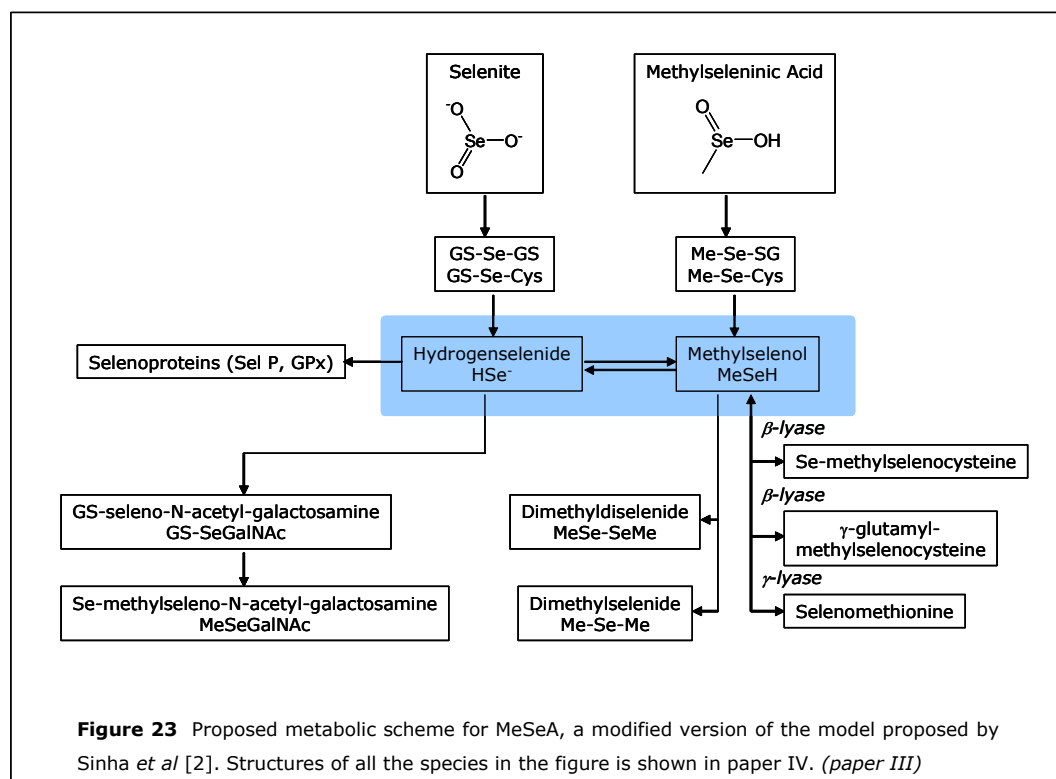
The retention times of the compounds, that were only detected in the short time incubations, matched the retention time of synthesized S-(MeSe)Cys and S-(MeSe)SG, respectively. As the S-Se metabolites were only observed in limited amounts it was not possible to prove their identity by LC-ESI-MS. Formation of these S-Se species would be expected as they are formed spontaneously upon reaction of MeSeA with Cys and GSH (*paper II*), that are present in large amounts in hepatocytes[51].



The MeSeA metabolism was proposed by Sinha *et al*[2] to be a series of reductions by glutathione to S-(MeSe)SG and further to methylselenol (MeSeH). Methylselenol then undergoes oxidation or methylation to form the volatile species dimethyldiselenide (MeSe-SeMe) or dimethylselenide (MeSeMe) respectively. As mentioned earlier, the formation of these volatile metabolites of MeSeA in a lymphoma B-cell line has been verified by GC-MS by Infante *et al*[72]. Furthermore, the preliminary results from the experiments with the membrane inlet EI-MS revealed, that the large amount of volatilised dimethyldiselenide also was observed from an aqueous mixture of MeSeA and GSH. Hence, dimethyldiselenide was not a product of hepatocyte metabolism. It was rather a product of a series of spontaneous reactions initiated by the reduction of MeSeA by GSH (*results not published*).

It is now clearly established that Se-MeSeCys and SeMet are derived from the metabolism of MeSeA in isolated rat hepatocytes. Hence, this observation should be included in metabolism models. As dimethylselenide and dimethyldiselenide are considered end products of selenium metabolism, there is reason to believe that MeSeA is metabolized through different pathways - one that leads to formation of volatile species, which may partly be due to spontaneous reduction by GSH and Cys, and pathways that lead to formation of Se-MeSeCys and SeMet and probably other not yet identified selenium species (figure 23). Selenoproteins and selenosugars are included in the metabolic pathways of MeSeA, as Suzuki *et al.* observed selenium of isotopically labelled MeSeA

origin in selenoproteins (extracellular glutathione peroxidase and selenoprotein P) and selenosugar in the rat[73,74].



When MeSeA is used as a model compound for monomethylated selenium species in cancer research, caution must be taken not to misinterpret mechanistic findings caused by the initial reduction steps in MeSeA metabolism that are not relevant to Se-MeSeCys metabolism. *In vitro*, the initial reduction of MeSeA could result in depletion of intracellular GSH and thereby mimic a state of oxidative stress leading to activation of cancer chemopreventive mechanisms[75]. *In vivo*, however, MeSeA is reduced instantaneously by ubiquitously present thiol compounds i.e. luminal GSH when orally administered or in red blood cells after *iv* injection. Thus, intracellular GSH depletion in the target cancer cells will not occur. An important study regarding this different behavior *in vivo* and *in vitro* was published by Ip *et al*.[64]. They compared the cancer chemopreventive effect of Se-MeSeCys and MeSeA *in vitro* (mammary cancer cell lines) and *in vivo* (chemically induced mammary cancer in the rat) and found lack of congruency between the *in vitro* and *in vivo* results. Cell culture data showed that MeSeA was more potent in inhibiting growth and in inducing apoptosis, while this difference in efficacy disappeared *in vivo*. Also Shen *et al*.[68] related intracellular GSH to MeSeA induced apoptosis and demonstrated GSH depletion in hepatoma cells treated with MeSeA.

The finding that Se-MeSeCys is a metabolite of MeSeA in both healthy and cancer cells, indicate that MeSeA may not be a relevant model compound for Se-MeSeCys after all. Hence, there is no rationale in treatment with a precursor to methylselenol that in it self is metabolised into another precursor of methylselenol.

It is important to notice that the results are obtained in models of epithelial cells from pigs and hepatocytes from rats. The in vitro – in vivo correlation as well as the animal – human correlation has not been investigated and is only speculative.

5 Conclusion

SeMet and Se-MeSeCys were not decomposed when subjected to *in vitro* gastrointestinal digestion. In two digestion models, selenite reacted differently; in one model selenite was extensively decomposed and in the other it was stable. Therefore SeMet, Se-MeSeCys and selenite are relevant compounds for intestinal metabolism studies.

Selenite and MeSeA were completely reduced by Cys and GSH in the gastrointestinal tract and will not be bioavailable in their intact forms. The labile reduction products of selenite; GS-Se-SG and Cys-Se-SG were identified; however the degradation products were not detected as they were either volatilized or associated to insoluble cellular debris. Also the intermediate reduction products of MeSeA; S-(MeSe)Cys and S-(MeSe)SG were observed in intestinal epithelial cell homogenates. The identity of the former was established by molecular MS whereas the identity of the latter was tentatively established by chromatographic coelution with a standard.

A new product, GS-Se-Se-SG in the series of reductions of selenite by glutathione was identified by molecular MS in an aqueous mixture of selenite and GSH.

In hepatocytes, MeSeA was extensively metabolised and about 75 % of the dosed selenium was volatilized. Besides being spontaneously reduced by Cys and GSH, MeSeA was metabolised into the selenoamino acids Se-MeSeCys and SeMet. The selenoamino acids were excreted from the hepatocytes into the cell medium. Preliminary experiments indicated that MeSe-SeMe constitutes a significant part of the volatilized selenium and also that it was produced from the spontaneous reduction of MeSeA by GSH. Furthermore, the intermediate methylselenylsulfides S-(MeSe)Cys and S-(MeSe)SG of the spontaneous reduction of MeSeA by Cys and GSH were detected in the incubation medium of hepatocytes incubated with MeSeA.

In conclusion, selenite and MeSeA are highly susceptible to spontaneous reduction by thiols. The series of reductions results in formation of a variety of selenium compounds and all of these are still not identified. The physiological relevance of the spontaneously formed selenium compounds has to be investigated.

The findings in the intestinal and hepatic models combined indicate that MeSeA may not be a relevant model compound for Se-MeSeCys in cancer research, as the two compounds are metabolised by different pathways and MeSeA is metabolised into Se-MeSeCys.

It is important to notice that the results are obtained in models of epithelial cells from pigs and hepatocytes from rats. The in vitro – in vivo correlation as well as the animal – human correlation has not been investigated and is only speculative.

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Appendices

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